Evaluation of Human Fetal Membrane Sealing

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Summary

Operative fetoscopy on the fetus and the placenta during pregnancy has become more and more a therapeutic option due to the strong progress in fiber endoscopes. Nevertheless, medical invasions into the amniotic cavity using needles or fetoscopes generate a defect in the fetal membranes and pose a significant risk of persistent leakage as well as preterm premature rupture of fetal membranes (referred to as iatrogenic PPROM; iPPROM). The risk for iPPROM after fetoscopic procedures has been reported to range from 6 to 45 %, depending on the procedures applied. The associated morbidity and mortality may compromise the expected benefit of the intervention and does strongly limit the developing field of prenatal fetal surgery.

Increasing efforts have been concentrated on preventive plugging of fetoscopic access sites. Nevertheless, none of these treatments have yet been translated into clinical practice. Accordingly, there is an unmet need for biocompatible, non-toxic and durable sealants for fetal membrane repair.

The objective of the present thesis is the investigation of new potential treatment options that allow the prophylactic plugging or sealing of fetal membrane lesions after fetoscopic interventions. In order to design appropriate sealing strategies, the knowledge of mechanical and biological properties of the fetal membranes is essential. Thus, we concentrated as a first step on the characterization of biophysical parameters of intact fetal membranes. We established a novel inflation device, together with the group of Prof. Edoardo Mazza from the Department of Mechanical & Process Engineering, ETHZ. This device allows studying the mechanical behaviour of the intact fetal membranes under close to physiological conditions. We performed equi-biaxial stretching to define the fetal membrane's strength and the deformation capacity. I established a protocol based on the hydroxyproline estimation to measure the total collagen content of the fetal membrane tissue. Furthermore, elastin was determined and the biochemical constituents were related to the biomechanical parameters, resulting from biaxial inflation testing. A trend towards a higher pressure resistance with higher collagen content was observed. The biochemical data was affected by uncertainties related to large inhomogeneities in fetal membrane tissue.

In the second part, potential candidate glues were evaluated, to test their sealing performance on uniform material. A standardized method was followed to compare the results in a simplified way. We demonstrated that mussel glue, a 4-arm PEG (Polyethylene Glycol) that is

functionalized by terminal catechol groups, is able to achieve relevant intrauterine pressure levels. Additionally, mussel mimetic glue showed a high deformation capacity.

In a third part, the in vitro stability of two possible liquid sealants, mussel glue and fibrin glue, were elaborated in the presence of defined proteolytic enzymes. Mussel glue was identified as a durable sealant, whereas fibrin glue showed degradation within short time. In additional experiments on punctured semi-wet fetal membranes, I showed that mussel glue performed better than fibrin glue corresponding pressure resistance and sealing performance.

In conclusion, the data presented herein demonstrate that mussel glue, a new promising tissue sealant that even binds to semi-wet fetal membrane tissue, can be used as an efficient injectible sealing material for fetal membrane repair. Nevertheless, in vivo evaluations are necessary to confirm the applicability of mussel glue for the prophylactic sealing of fetoscopically punctured fetal membranes.

Zusammenfassung

Die operative Fetoskopie am Fetus und der Plazenta während der Schwangerschaft wird durch den starken Fortschritt in der Faserendoskopie vermehrt als therapeutischer Ansatz eingesetzt. Medizinische Eingriffe in die intrauterine Fruchthöhle mit Nadeln und Fetoskopen generieren einen Defekt in die Fruchtblase und führen somit zu einem signifikanten Risiko für einen anhaltenden Fruchtwasseraustritt sowie einen verfrühten vorzeitigen Blasensprung (sogenannt iatrogenen PPROM; iPPROM). Fetoskopische Eingriffe führen in 6 bis 45 % der Fälle zu Komplikationen durch iPPROM und hängt vom angewendeten operativen Verfahren ab. Die damit assoziierte Morbidität und Mortalität beeinträchtigt den erwarteten Vorteil eines solchen Eingriffes und limitiert den sich stark entwickelnden Bereich der fetalen Chirurgie.
Bestrebungen konzentrieren sich auf einen präventiven Verschluss von fetoskopischen Eingriffsstellen. Dennoch wurde bis jetzt keine der Behandlungen routinemässig in die Klinik eingeführt. Folglich besteht eine grosse Nachfrage für biokompatible, nicht toxische und langzeitstabile Verschlussmaterialien für die Reparatur von Fruchtblasendefekten.
Die Zielsetzung der vorgelegten Arbeit ist die Untersuchung eines neuen potentiellen Verschlussklebers zur präventiven Behandlung und zum Verschluss von Fruchtblasen-Läsionen nach fetoskopischen Interventionen. Um geeignete Verschlussmethoden zu entwickeln sind Grundkenntnisse der mechanischen und biochemischen Eigenschaften des Fruchtblasen-Gewebes unerlässlich. Daher konzentrierten wir uns in einem ersten Schritt auf die Charakterisierung der biophysikalischen Parameter von intakten Fruchtblasenproben. Wir etablierten zusammen mit der Gruppe von Prof. Edoardo Mazza vom Departement Mechanik der ETH Zürich ein neues biomechanisches Aufblas-Gerät, das uns erlaubt, das mechanische Verhalten von intakten Fruchtblasenproben unter annähernd physiologischen Bedingungen zu erforschen. Um die Stärke und die Deformierbarkeit des Fruchtblasen-Gewebes zu definieren, führten wir equi-biaxiale Dehnungsversuche durch. Um den Kollagengehalt dieses Gewebes zu bestimmen, etablierte ich ein Protokoll basierend auf der Hydroxyprolin-Messung. Ausserdem wurde der Elastingehalt mittels eines biochemischen Kits detektiert. Die ermittelten biochemischen Gewebebestandteile wurden mit den biomechanischen Parameter, die aus den Druck- und Deformationswerten der mechanischen Tests berechnet wurden, in Relation gebracht. Ein Trend zu höheren Druckwiderständen bei gesteigertem Kollagengehalt wurde beobachtet. Die biochemischen Daten von Elastin und Kollagen wurden von Messunsicherheiten beeinträchtigt, die auf Schwierigkeiten der Quantifizierung von Elastin und Kollagen in Geweben aufmerksam machen. Zudem weist das Fruchtblasen-Gewebe starke Inhomogenität auf, die dessen Evaluation erschweren.

In einem zweiten Teil evaluierten wir neue potentielle Verschlusskleber hinsichtlich der Abdichtungsleistung auf normiertem einheitlichem Membranmaterial. Zur Vereinfachung der Resultate verwendeten wir eine standardisierte Methode. Wir konnten aufzeigen, dass der Muschelkleber, ein 4-armiges PEG (Polymerethylenglykol) das durch randständige Katecholgruppen funktionalisiert wurde, intrauterin relevante Druckwerte erreichen kann. Zusätzlich wies der Muschelkleber eine hohe Deformierbarkeit auf.

Im dritten Teil der Arbeit wurde die in vitro Stabilität des Fibrinklebers und des Muschelklebers in Anwesenheit von definierten Enzymen getestet. Dabei wurde der Muschelkleber als permanentes Verschlussmaterial erkannt, im Gegensatz zum Fibrinkleber, der innerhalb kurzer Zeit durch proteolytische Enzyme abgebaut wurde. In Experimente an punktierten halbfeuchten Fruchtblasenproben konnte ich deutlich aufzeigen, dass der Muschelkleber im Vergleich zum Fibrinkleber eine bessere Abdichtungsleistung und höhere Druckwiderstände aufbringen konnte.

Abschliessend zeigen die hier präsentierten Daten, dass der Muschelkleber ein neuer vielversprechender Gewebekleber darstellt, der sogar im halbfeuchten Zustand an Fruchtblasengewebe bindet und effizient als prophylaktischer Verschluss von Fruchtblasen-defekten eingesetzt werden kann. Dennoch sind in vivo Ermittlungen unerlässlich, um die Applizierbarkeit des Muschelklebers für den prophylaktischen Verschluss von fetoskopischen Fruchtblasendefekten zu bestätigen.

Table of contents

Abbreviations

ABS	Amniotic band syndrome
AFL	Amniotic fluid leakage
DAM	Decellularized amnion plug
DOPA	3, 4-dihydroxyphenylalanine
E	Elastic modulus, Young's modulus
F	Force
FM	fetal membranes
G, Gly	Glycine
GAG	Glycosaminoglycan
iPPROM	Iatrogenic preterm premature rupture of the membranes
K	Elastic constant
MMP	Matrix-metallo-proteinase
N, Asn	Asparagine
P	Pressure
PEG	Polyethylene glycol
PROM	Premature rupture of the membranes
PPROM	Preterm premature rupture of the membranes
R, Arg	Arginine
ROM	Rupture of the membranes
SEM	Scanning Electron Microscopy
sPPROM	Spontaneous preterm premature rupture of the membranes
T	Tension
TEM	Transmission Electron Microscopy
WHO	World Health Organization
ZAM	Zone of altered morphology
ε	Strain
λ	Stretch
σ	Stress
σ_F	Failure Strength / Tensile Strength
r	Radius
t_0	initial thickness
ω_0	initial width

1. Introduction

1.1. Early embryonic & fetal membrane development

Early human development is a tightly orchestrated process of cell division (mitosis), cell migration, programmed cell death, differentiation, growth, and cell rearrangement in order to transform the fertilized oocyte, into a highly organized multicellular organism, the human being. In order to survive during the intrauterine life, the embryo must maintain a connection to the body of the mother for acquiring oxygen and nutrients. It must also avoid being rejected like a foreign body by the maternal immune system. To fulfil these requirements, the surrounding placenta and fetal membranes provide the contact interface between the embryo and the mother.

After fertilization, the zygote divides into several embryonic cells and approximately 3 days later, the inner cell mass differentiates from the outer trophoblast cell layer, see figure 1.1A, while the cleaving zygote travels along the uterine tube. The trophoblast is the forerunner of the placenta (embryonic part), while the inner cell mass forms the embryoblast, from which the embryo, umbilical cord and amnion are derived. The placenta as well as the chorion make up the feto-maternal interfaces and both are derived from the trophoblast and surround the cellular precursor of the embryo.

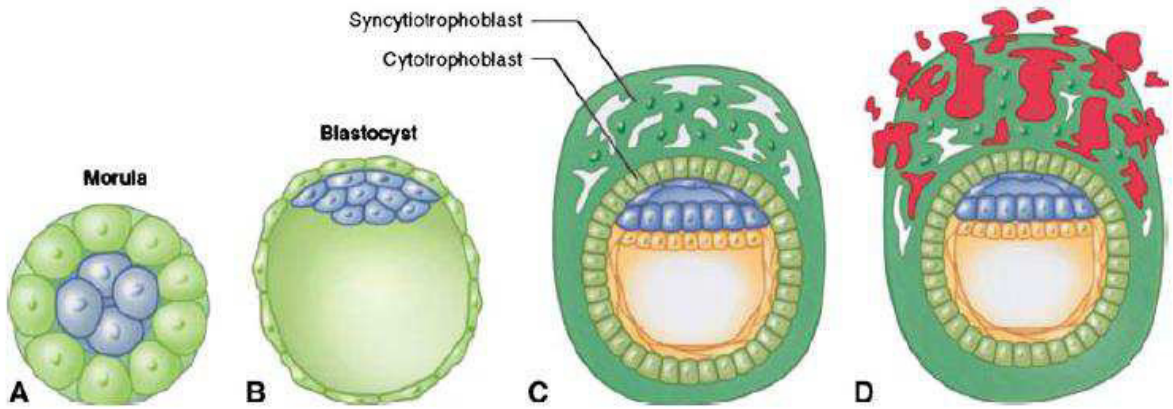

Figure 1.1 Early embryonic development A) The morula at 3 to 4 days after fertilization is characterized by an inner cell mass (blue) and an outer cell mass (green). B) During the blastocyst stage, the cells of the outer cell mass, the trophoblast, will form the placenta. C) Trophoblast differentiation occurs shortly after the blastocyst implants on the endometrium. The outer layer is the syncytiotrophoblast (dark green) and the inner layer is the cytotrophoblast (light green). D) As the blastocyst continues to invade the endometrium, lacunae form within the syncytiotrophoblast layer and contain maternal blood (red) (Ernst 2011).

During entering of the spherical blastocyst into the uterus, a fluid-filled space called the blastocystic cavity is developed, as presented in figure 1.1B. The blastocyst attaches to the endometrial epithelium and the trophoblast cells proliferate forming a double layer of an outer syncytiotrophoblast towards the maternal tissue and an inner cytotrophoblast, figure 1.1C (Sadler 2009). The syncytiotrophoblast invades the endometrial connective tissues by finger-like extensions; vacuoles (lacunae) begin to form which get filled with maternal blood as soon as the trophoblastic cells reach the maternal blood vessels, figure 1.1D (red). Thus, the beginning of the maternal circulation to the placental tissues is established at approximately 12 days (Sadler 2009; Schoenwolf 2009). Fluid can pass by diffusion, providing nutritive material to the embryo. The blastocyst gets fully implanted by the end of the second week and the uterine epithelium closes over the implantation site. Endometrial stromal cells transform into decidual cells (Baergen 2011).

The embryoblast cells, lining the inner surface towards the blastocystic cavity are amniogenic cells and thus exhibit the primordium of the amnionic epithelium. The so-called amnioblast cells develop in continuity with the cytotrophoblast shell surrounding the embryo. They separate from the embryoblast by a cleft as fluid collects between embryoblast and the neighbouring trophoblastic cells forming the amnion, which encloses the early amniotic cavity (see figure 1.2A). From the 7th or 8th day on, the amnion can be identified. At first, it is smaller than the blastocystic cavity but expands continuously; by the 8th week, the amniotic cavity encloses the embryo (figure 1.2 A-D) (Schoenwolf 2009).

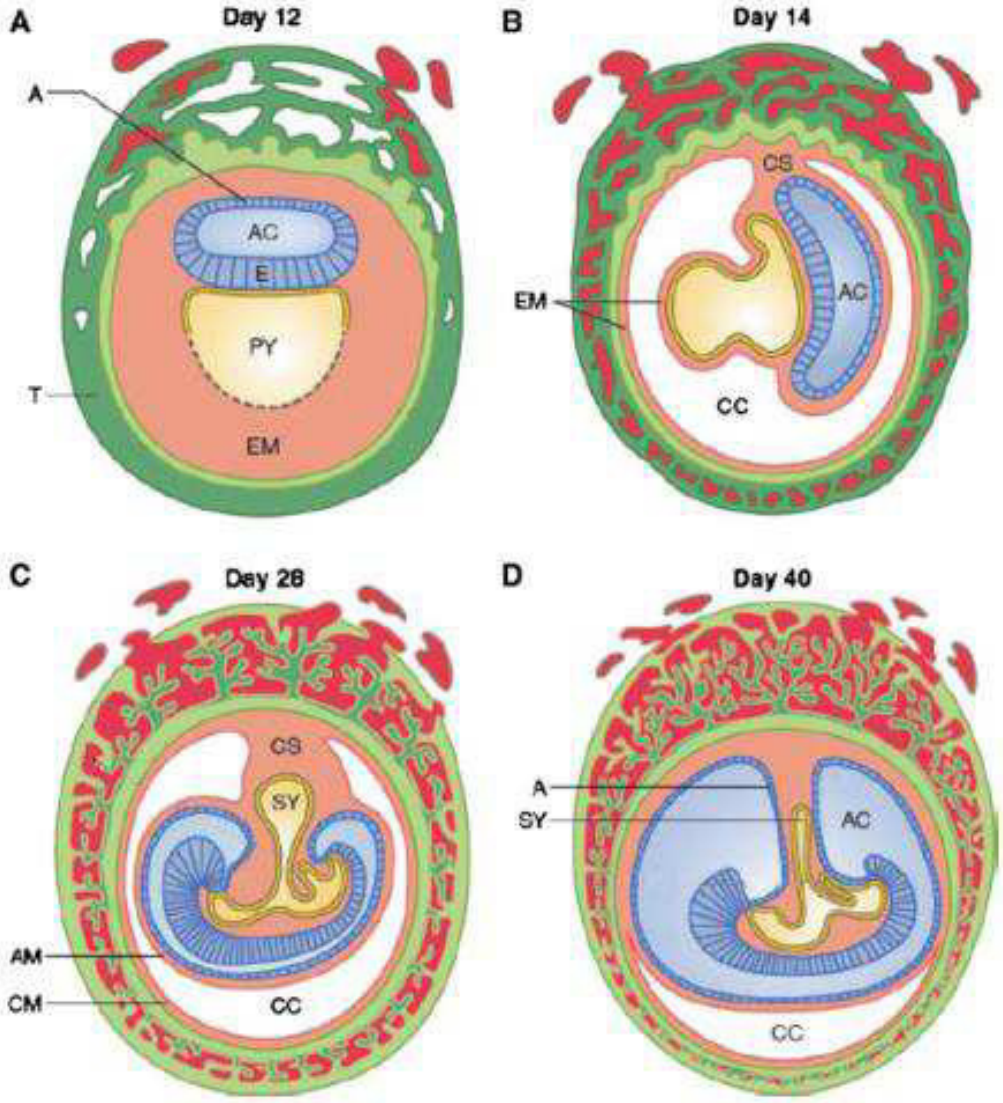

Figure 1.2 Formation of the amnionic cavity, chorionic cavity, and umbilical cord. A) Day 12. The amnionic cavity (AC) begins as a small cavity within the embryoblast layer (E). The cells lining the cavity will form the amnion (A). The cells of the hypoblast form the lining of the primitive yolk sac (PY). The extraembryonic mesoderm (EM) fills in the space between the two existing sacs (amnionic and primitive yolk sac) and the cytotrophoblast (T). B) Day 14. A cavity has formed within the extraembryonic mesoderm (EM), the chorionic cavity (CC). The extraembryonic mesoderm (EM) is present on the inner surface of the trophoblast surrounding the yolk sac and the amnionic sac (AC). The connecting stalk (CS) begins to develop. C) Day 28. As the amnionic sac expands to fill the chorionic cavity, the mesodermal tissues of the amnion (AM) and chorion (CM) come in close contact. The secondary yolk sac (SY) and connecting stalk (CS) are developing. D) Day 40. As the trophoblast develops and invades the endometrium, continued maturation of the amnionic (AC) and chorionic (CC) cavities that surround the connecting stalk continues while the embryo folds (Adapted according to (Ernst 2011)).

The hypoblast is a part of the primary endoderm, which differentiates and grows as a 2nd cavity inside the blastocyst cavity ultimately forming the primary yolk sac at around day 12, according to figure 1.2A (Hertig, Rock et al. 1956). The hypoblast and the embryoblast

(amnioblast) create a bilaminar cell layer, also called the embryonic disc as they will give rise to the three germ layers that form all the tissues and organs of the embryo. Two hemispheric cavities oppose each other: the amniotic cavity and the primary yolk sac and adjoin to the embryonic disc. The extra-embryonic mesoderm forms, filling the remaining blastocyst cavity between the two existing sacs (amnion and primitive yolk sac) and the cytotrophoblast, see figure 1.2A. The primary yolk sac gets displaced (and eventually degenerates) by further migration and forms the smaller definitive or secondary yolk sac (Schoenwolf 2009).

At day 14, a new cyst is produced within the extra-embryonic mesoderm, corresponding to the chorionic cavity, as figure 1.2B illustrates, and surrounds the amnion and the yolk sac. The embryonic bud is suspended by a connective stalk at the inside of this large balloon (chorionic sac). Exclusively, the connecting stalk is in direct contact to the extra-embryonic mesoderm and the cytotrophoblast, becoming the ultimate umbilical cord, linking the fetal and the placental circulations. The extra-embryonic mesoderm lines the inner surface of the trophoblast as well as the outside of the yolk and amniotic sac. With this development, both the amniotic and chorionic linings contain a mesoderm component, which will form the definitive connective tissue layers of the membranes (Ernst 2011).

Around day 28 of gestation, the yolk sac becomes integrated into the connecting stalk (figure 1.2C). The amniotic sac enlarges, fills out the chorionic cavity and finally provides the epithelial covering of the fetal skin as well as of the umbilical cord (figure 1.2C & D). The amniotic sac enlarges faster than the chorionic sac, thus the mesodermal tissues of the amnion and chorion come in close contact and fuse for the first time near the cord insertion site (Schmidt 1992). Further expansion leads to overall fusion of the amniochorionic or fetal membranes and is completed around the 12th week. However, fusion of the amnion and the chorion is never complete, and thus the two membranes can slide against each other (Baergen 2011). A potential space exists between the amnion and chorion connective tissue layers (intermediate spongy layer), which can be seen histologically as a gap, fluid-filled clefts or composed of loosely arranged collagen fibers, as illustrated in figure 1.3 (Ernst 2011).

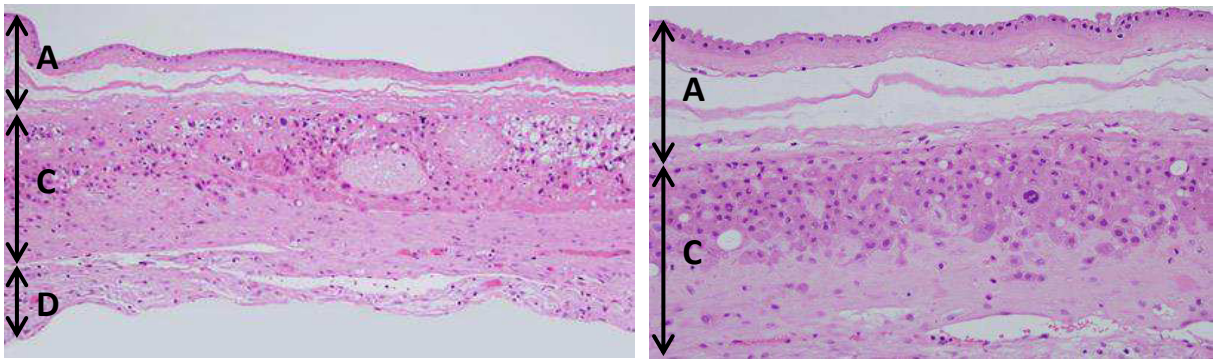

Figure 1.3 Fetal membranes at term. Left: The amnion (A) shows more cuboidal, epithelial cells. The chorionic (C) trophoblast is somewhat thicker. A clear distinction can be made between the decidual (D) cells with polygonal, pale cytoplasm and the more amphophilic cytoplasm of the chorionic trophoblast. (H&E, 10×). Right: Higher-power view of the membrane components. Note the potential space between the amnion and chorion with the spongy layer containing loose collagen fibers. Trophoblast cells of the chorion have variable sizes and shapes and occasional multinucleated cells can be observed. (H&E, 20×) (Ernst 2011).

Thus, amnion and chorion can be easily separated by gentle traction at delivery (Calvin and Oyen 2007). Embryologically, this space represents the remnant of the extra-embryonic mesoderm. The situation is different for the umbilical cord; the expanding amnion not only gets closely attached to the cord, but also firmly fuses with it and cannot be dislodged from the cord. The fetal membranes, consisting of the amnion (innermost membrane facing the amniotic fluid) and the chorion (facing the uterine wall), come into close contact with the uterine wall.

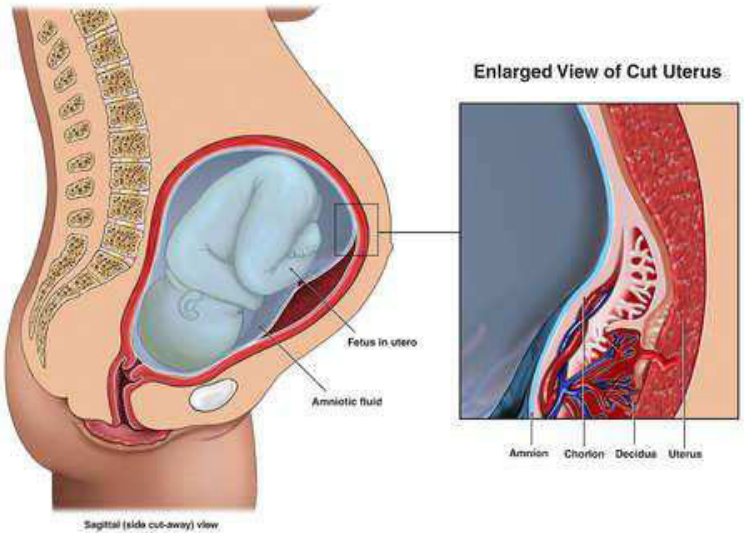

Figure 1.4 Sagittal image of the pelvis of a pregnant woman. The fully developed fetus is visible inside the amniotic cavity within the uterus (Health). In the enlarged view: amnion, chorion, decidua and the uterus are indicated.

Between the 15th and 20th week, the smooth chorion locally fuses with the decidual surface (maternal tissue that lines the uterus during pregnancy), thereby largely obliterating the uterine cavity. Finally, the chorion has contact with the decidual surface of the uterine wall over nearly its entire surface. The amazingly strong but soft membranes contain not only the fetus but also the amniotic fluid during the entire gestation as shown in figure 1.4. Despite the considerable expansion of the amniotic cavity during fetal growth, the structure of the fetal membranes generally remains constant from the 4th month until term (Schmidt 1992).

However, it is still unknown how the expansion of amnion is achieved without additional cell division and growth of the cells as well as matrix production.

1.2. Structure of fetal membranes

Histological sections of the human fetal membranes are composed of three main tissue layers that surround the amnionic sac: the fetally derived amnion and chorion, as well as the maternally derived decidua, see figure 1.3. Over the gestational period, all three layers remain with only minor changes in thickness or epithelial appearance (Ernst 2011). The total thickness of the fetal membrane has been described between 0.796 ± 0.159 mm and 0.818 ± 0.176 mm at the placental margin (Schmidt 1992). The normal organization and the microscopic anatomy of the human chorion and amnion has been reported in detail in the classic work provided by Bourne in the 1960s where transmission and scanning electron microscopy (TEM and SEM) was applied (Bourne 1960). The representation in figure 1.5 names the different layers of the fetal membranes.

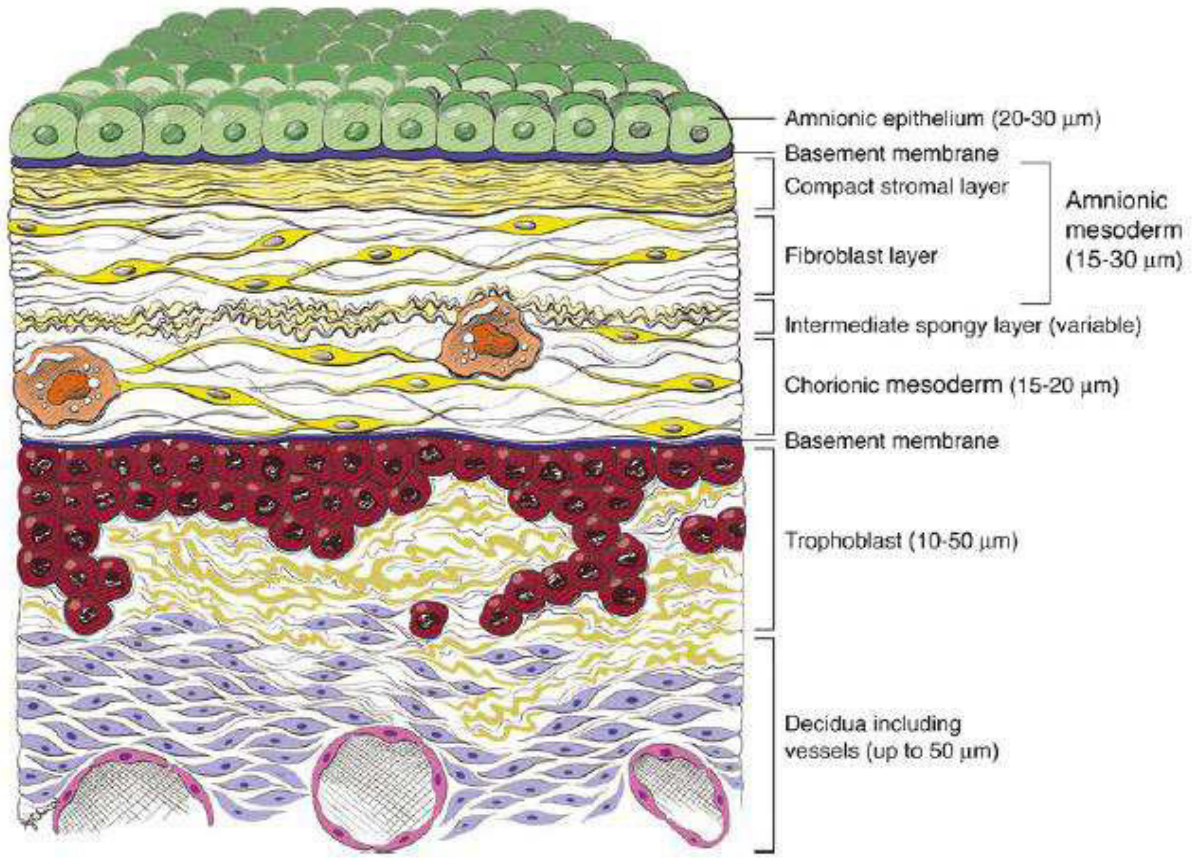

Figure 1.5 Detailed drawing depicting the layers of the fetal membranes. Drawing is not to scale (Baergen 2011).

Identification of the structural characteristics of fetal membranes before and after labour onset may help in understanding the mechanism in their rupture and fracture properties.

1.2.1. Amnion

Depending on the position within the amniotic sac, the following three parts of the amnion can be distinguished: the placental amnion, the amnion enclosing the umbilical cord and the reflected amnion where the embryonic sac is opposed to the uterine wall. The three regions appear different in structure by visualization. For our work, the attention is concentrated on the reflected part of the amnion.

Macroscopically, the amnion is a thin (accounts for only 20 % of the fetal membrane thickness) and translucent structure. It can easily be separated from the underlying chorion as it never truly fuses with the chorion and it is only passively attached by the internal pressure of the amniotic fluid (Baergen 2011). The amnion does not possess blood vessels and obtains required nutrients and oxygen for the amnion cells supplied directly by diffusion from the surrounding amniotic fluid, the fetal surface vessels and/or from the underlining decidua (Parry and Strauss 1998).

The thickness of amnion varies greatly due to the alterations in the amount of mucin and fluid contained in the spongy layer; it has been reported to be in the range of 80 - 120 µm (Polzin and Brady 1991).

The amnionic epithelium, the innermost amnion layer is directed towards the fetus and is contacting the amniotic fluid. It is composed of a single layer of simple, squamous to cuboidal cells, with apical microvilli and is attached to the basement membrane (Aplin, Campbell et al. 1985). Taller, columnar cells are usually present near the insertion of the membranes at the placental margin, whereas flatter cells are generally present in the periphery (Baergen 2011). Observed from the top, the cell arrangement shows a rectangular to hexagonal surface (Bourne 1960). In cross sections the cells are almost square with a convex roof. The height of the amnionic epithelial cells was described by various investigators resulting in variable ranges. Explanations for these differences are irregularities; the structure of cells depends upon their functional state; according figure 1.5 Baergen et al. presented the epithelial cell height between 20 - 30 µm, beside others defining it between 4 - 17 µm (Schmidt 1963; Hempel 1972). The height might be influenced additionally by the preparation in customary paraffin embedding, where the sections are expected to shrink by 20 – 25 % (Schmidt 1992). The amnionic epithelium secretes collagen type I, III and IV and non-collagenous

glycoproteins, such as laminins, nidogen and fibronectin, (Bourne 1960; Parry and Strauss 1998), which form the approximately 1 µm thick basement membrane providing cell attachment sites (Schmidt 1992).

The compact stromal layer, which remarkably varies in thickness (Bryant-Greenwood 1998), is strongly adherent and cannot be separated (Bourne 1960) from the basement membrane. It is a cell-free homogenous layer compacted of microfibrils. The collagens type I, III and V as well as elastic fibers of the compact layer are secreted by the mesenchymal cells in the fibroblast layer underneath the compact layer (Strauss 2012). They form the main load bearing fibrous skeleton of the amnion. The major tensile strength of the amnion is provided by the collagens being arranged in one direction, i.e. parallel to the amnionic epithelium (Schmidt 1992). The compact and the fibroblast layer are difficult to distinguish on histologic section.

The fibroblast layer lies beneath the compact layer, the most complex and thickest of the amniotic layers, which is composed of a cellular network within a loose network of collagens with islands of non-collagenous glycoproteins, proteoglycans and typ III collagen. In this layer fibroblasts and macrophages (so-called Hofbauer cells) have been identified (Bourne 1960).

The intermediate spongy layer, also called intermediate layer as it lies between amnion and chorion, is rich in proteoglycans and hyaluronan which by uptake of large amounts of water swells and forms a viscous layer that allows the sliding of the amnion over chorion (Bryant-Greenwood 1998; Meinert, Eriksen et al. 2001). By this mechanism, the spongy layer permits to absorb physical stress and has been proposed to provide a spontaneous short-term mechanical repair and defect-closure system (Behzad, Dickinson et al. 1994; Gratacos, Sanin-Blair et al. 2006). In addition, coiled fibrous structures are found in the direct relation to the chorionic side, helping to adjust during lateral tension at this shear surface between amnion and chorion (Ockleford, Bright et al. 1993). Occasionally, potential spaces can be seen histologically in the spongy layer between the amnion and chorion (Benirschke K 2006). The space is composed of loosely arranged collagen fibers (see figure 1.4) that sometimes have an oedematous appearance and contain scattered fibroblasts and macrophages.

1.2.2. Chorion

The chorionic layer is composed of epithelial and connective tissue layers as the amnion. However, compared to the amnion, the layers in the chorion are reversed in orientation. The

chorionic mesoderm (connective tissue layer) is adjacent to the connective tissue layer of the amnion with the basement membrane layer underneath. The soft chorion is more cellular and less organized compared to the amnion (Calvin and Oyen 2007). The chorion itself is described to be avascular (Benirschke K 2006), thus, all fetal-derived tissues in the extra-placental membranes are avascular (Schmidt 1992). Although the chorion is thicker than the amnion, in previous studies the amnion achieved greater tensile strength and viscoelasticity (Oxlund, Helmig et al. 1990; Parry and Strauss 1998). According to Schwarzfurtner et al., amnion and chorion represent equal collagen content (Schwarzfurtner, Huttegger et al. 1986). Its lesser strength may be the result of the looser structure and the sparse cross-connections of the fibril bundles (Bourne 1960). The chorion is composed of three tissue layers as described next.

The chorionic mesoderm (connective tissue or reticular layer) consists of fibroblasts and macrophages, which are embedded in an inhomogeneous reticular meshwork. Secreted fibres within this layer are collagen type I, III, IV, V and VI, which extend deeply into the trophoblastic layer and connect the different chorionic structures (Parry and Strauss 1998). The chorionic mesoderm is slightly thicker than the mesoderm of the amnion but has a similar appearance of collagen and elastic fibers with occasional macrophages.

The chorionic basement membrane is also called pseudo-basement membrane as it is a narrow band of reticulin tissue composed of collagen type IV, fibronectin, and laminin (Bourne 1960).

The trophoblast (epithelial) layer of the chorion has several layers of trophoblastic cells and the thickness of the trophoblastic layer is variable, its depth is indicated from 10 - 50 µm, presenting 2 - 10 cells in a trophoblast arrangement, see figure 1.5. The trophoblast layer is in direct contact with the underlying maternal decidual layer of the uterus. Histologically, it is difficult to unambiguously determine where the cytotrophoblast ends and where the decidua starts.

1.2.3. Decidua

The decidua, representing the modified endometrium, is the only maternal component of the membranes. It is the only vascularized tissue of the fetal membranes and has both superficial and deep vascular components. The maternal vasculature in the decidua does not undergo invasion and remodelling by the trophoblast (Benirschke K 2006).

1.2.4. Extracellular matrix components of the fetal membranes

The extracellular matrix (ECM) includes the interstitial matrix, present between cells i.e. inter-cellular spaces, and the basement membrane. It consists of a complex cell-secreted network of proteins, glycoproteins and proteoglycans, that at the same time provides structural support, acts as a compression buffer against mechanical and chemical stress, segregates tissues from one another as well as allows interactions among each other. The molecular composition and thus both mechanical and signalling properties are dynamically regulated during tissue formation and remodelling by secretion as well as proteolytic modifications. The ECM controls cellular processes such as cell migration, cell adhesion, spreading, cell shape and apoptosis through interactions with cell, that vary as example with the ECM density. The major types of macromolecules present in an average ECM are fibrous proteins, such as collagen, elastin, fibronectin, and laminin, as well as proteoglycans (protein-saccharide complexes), composed of glycosaminoglycan chains and hyaluronan covalently bound to the core protein (R. Lanza 2007).

Basement membranes are sheet-like depositions of specialized ECM. They are found at the basal surfaces of epithelial and endothelial cells, they surround all muscle, fat cells, the central nervous system, and peripheral nerves. Basement membranes are typically composed of collagen, laminin, nidogen and sulfated proteoglycans.

The structure and function of ECMs are divers and adapted to tissue-specific function. ECM turnover and homeostasis are highly regulated and the catabolism is due to the action of a specific class of proteolytic enzymes known as matrix metalloproteinases (MMP's) (Woessner 1991).

Regarding the fetal membranes, a balance between the synthesis and the degradation of membrane components is physiologic throughout gestation. The two principle mechanisms involved in the degradation process are apoptosis in the cellular compartment as well as MMP's in the extracellular matrix. Regulation of MMPs depend on factors increasing their expression (cytokines) and factors decreasing their activity, such as tissue inhibitor of metalloproteinases (TIMP's) (Benirschke K 2006).

Tissue integrity, mechanical strength and elasticity are of central interest in the context of fetal membranes, thus the following paragraph describes the main components conferring these functions. Based on their well-recognized mechanical function in fetal membranes connective tissue, the extracellular matrix components such as collagen, elastin and proteoglycans are of especial interest in the present work.

Collagens

Collagens constitute a large family of proteins that represent the major proteins in mammalian tissue (about 25 %,(Kielty 2002)). There are 28 genetically distinct types of collagen, that assemble into a variety of supramolecular structures including fibrils, microfibrils, and network-like structures which have been identified so far. Collagens are mainly found in the extracellular matrix as fibrillar proteins to give structural support to resident cells and provide high tensile strength at tissue level. They are not only highly abundant in tissues such as tendon, ligament and skin, cartilage, and bone but are also abundant in cornea, blood vessels, the gut, and the intervertebral disc. Collagen types differ in their amino acid sequence but also in their spatial confirmation conferring specific functional properties to them. Fibroblasts mainly secrete collagen, but also epithelial cells (Mehats, Schmitz et al. 2011).

Fibrillar collagen molecules are the major components of collagen fibrils. They are characterized by a right-handed triple helix which is formed out of three rod like molecules, the subunits called α-chains which are the left-handed polypeptide chains, see figure 1.6. One α-chain is about 1000 amino acids long. The stabilization of the triple helix with in turn twisted α-chains can only be achieved by amino acids without side chains. Therefore, only glycine residues can be accommodated in this position. Due to this fact, collagens are characterized by the triplet repeats glycine (Gly)-X-Y where X and Y frequently are hydroxyproline or hydroxylysine, that can assemble into stable triple helical fibers, the so-called procollagens (Leppert and Yu 1991). Proline or hydroxyproline constitutes about 1/6 of the total amino acid sequence of collagen.

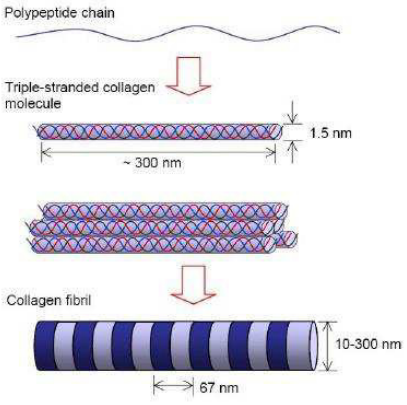

Figure 1.6 Schematic diagram of Type I collagen fibril structure. Three amino acid chains (polypeptide chains) form triple helix molecules (pro-collagens). They associate and form thick collagen fibrils by lateral assembly (Instruments 2009).

Higher order macromolecules are generated by posttranslational modifications forming aggregates which are stabilized by intra- and intermolecular cross-links. The triple helical rod like domains polymerizes by end-to-end (linear) and/or lateral assembly (figure 1.6.) from

immature pro-collagens to mature complex and cross-linked macrofibers. The enzyme lysil-oxidase mediates intra- and inter-molecular covalent cross-linking to stabilize the collagen fibers. The growth in length and diameter as well as covalent cross-linking increases the fetal mechanical strength and tensile properties (Prévost 2006).

Different collagens conduct different functions in the fetal membranes as reported earlier and do undergo differential collagenolysis controlled by hormones before and at parturition. Throughout pregnancy, the permanent turnover and remodelling process is crucial to accommodate the increasing volume and tension as gestation progresses (Parry and Strauss 1998).

Collagen type I and III, together with smaller amount of types IV, V and VI form the major structural framework of the fetal membrane ECM (figure 1.7) (Aplin, Campbell et al. 1985; Leushner and Clarson 1986; Prévost 2006). Collagen I, III and V belong to the same family of fibrillar collagens, for which aggregates in a staggered arrangement form fibrillar structures, as presented in figure 1.6. Collagen type I and III are mainly responsible for the mechanical integrity of the amnion by forming thick fibre bundles (Malak, Ockleford et al. 1993). Collagen type I forms about 40 nm thick fibrils and collagen type III around 10 - 12 nm microfibrils (Schmidt 1992). These collagens are connected to the basement membrane by collagen filaments of type V and VI (Parry and Strauss 1998). Type IV collagen molecules are the major component of the basement membrane in amnion and chorion (Prévost 2006) and provides anchoring function for other non-collagenous proteins (laminin, entactin and proteoglycan). They play a major role in the development and maintenance of the ECM (Malak, Ockleford et al. 1993).

Determinations of the total collagen content in the fetal membranes by hydroxyproline measurements showed that the collagen content of amnion is around twice of that of chorion, around 33 – 52 % versus 12 - 15 % (Halaburt, Uldbjerg et al. 1989; Hampson, Liu et al. 1997; MacDermott and Landon 2000; Meinert, Eriksen et al. 2001). During the last 8 weeks of pregnancy, decreased collagen content was observed (Parry and Strauss 1998).

The collagen fibres' contribution to the mechanical tissue resistance is estimated primarily at larger strains during reorientation and straightening in the direction of loading from the initial "crimped" state. Therefore, at high strain higher stiffness of collagen dominates compared to the more compliant elastin (Jabareen, Mallik et al. 2009).

Fortunato, Menon et al. reported biochemical studies on human fetal membranes indicating a relation of decreased collagen content, altered collagen structure, and increased collagenolytic

activity to premature rupture (Fortunato, Menon et al. 1997). Key players in the collagen remodelling and turnover are MMPs and TIMPs as introduced in the ECM part. Types of MMPs, which have been found in the fetal membrane layers, are indicated in figure 1.7.

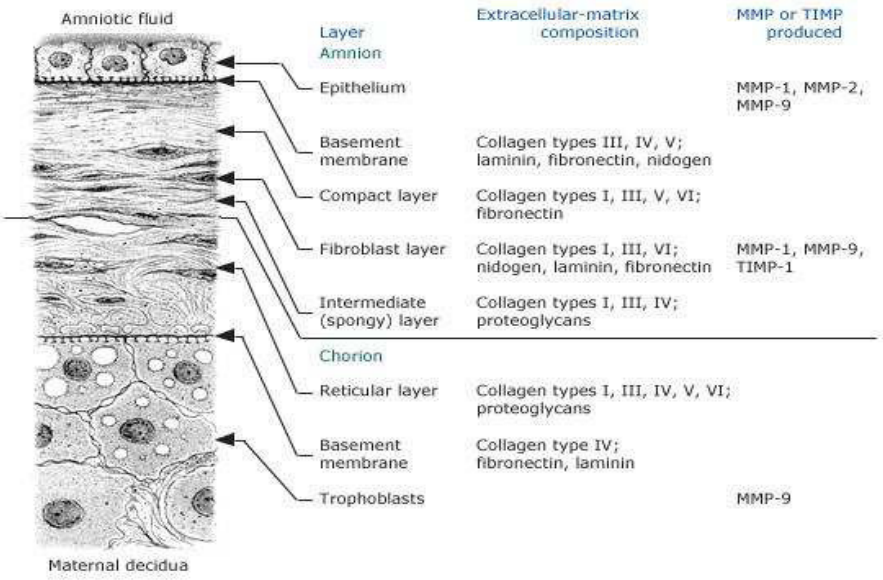

Figure 1.7 Extracellular matrix composition and enzymes in the different layers of human amnion and chorion (Parry and Strauss 1998).

Elastin

Elastin molecules correspond to complex insoluble proteins which are cross-linked to fibrillin-based microfibrils. Elastic fibers consist of the protein fibrillin and elastin which are build-up of simple amino acids such as glycine, valine, alanine, and proline (Kielty, Sherratt et al. 2002).

Elastin molecules are assembled from a soluble precursor protein tropoelastin. In a reaction catalyzed by lysyl oxidase, many soluble tropoelastin molecules are linked together to make an extensive insoluble, durable cross-linked array forming an elastin fiber. The amino acid responsible for these cross-links is lysine (R. Lanza 2007). The insoluble elastin molecules in tissues are very difficult to isolate (Christiansen 1991).

Elastin is important in load-bearing tissues of the body of vertebrates and abundant in locations where mechanical energy needs to be stored. In contrast to collagens being responsible for the high tensile strength, elastin molecules provide elasticity so that they can stretch when needed and afterwards return into their original state. Elastin fibres present impressive elastic properties with an extensibility of about five times that of a rubber band.

Elastin and fibrillin are associated with tissues capable of undergoing large strains without rupture, e.g. blood vessels, lung and skin (Christiansen 1991).

Concerning fetal membranes, particularly the mesenchymal and reticular tissue layers, the compact layer of amnion and chorion, plus the chorionic cytotrophoblast were demonstrated to contain elastin (Malak and Bell 1994; Hieber, Corcino et al. 1997). Elastin microfibrils orient parallel to each other and form bundles lying in parallel to the plane of the fetal membrane stretch. These proteins, having mechanical properties analogue to rubber corresponding the deformation, confer extensibility, flexibility and compliance to the fetal membranes (Bryant-Greenwood 1998).

Proteoglycans

Proteoglycans are extracellular matrix proteins composed of a protein core, to which a special class of long-chain polysaccharides, called glycosaminoglycans (GAGs), are covalently attached. A proteoglycan unit consists of one core protein, forming a long polypeptide strand with one or more GAG chain(s) of various lengths e.g. repeating disaccharides. The general structure of proteoglycans is illustrated in figure 1.8.

These long chains of linear carbohydrate polymers are negatively charged under physiological conditions due to the occurrence of sulfate and uronic acid groups. This results in the attraction of large water quantities by the Donnan effect (Leppert and Yu 1991).

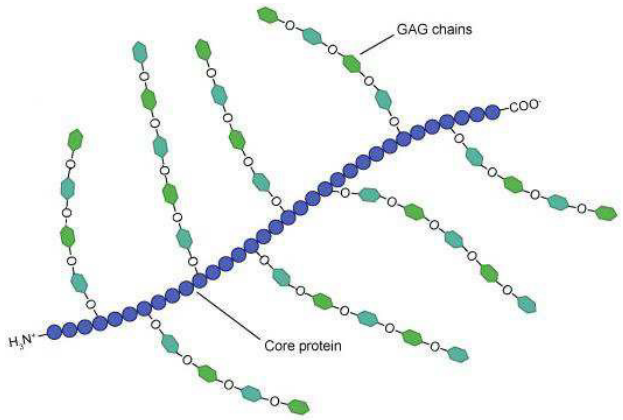

Figure 1.8 Proteoglycan structure with core protein and GAG chains.

Proteoglycans form macromolecular complexes with other proteoglycans, with hyaluronan (the simplest GAG) and fibrous matrix proteins (such as collagen). Thus, they occupy a large volume to fill space within the tissue. Proteoglycans contribute to the structural and mechanical stability of connective tissues, make the tissue resistant to compression, serve as

lubricants by their unique gel-like properties but also play an important role in cellular growth and differentiation. Individual functions of proteoglycans can be attributed to either the protein core or the attached GAG chain. Proteoglycans range from large hydrophilic space filling complexes such as aggregan and versican with the carbohydrate component hyaluronan (Meinert, Eriksen et al. 2001), to the cell surface receptors such as syndecan.

Concerning fetal membranes, proteoglycans are structural components, which are of importance in generating tensile strength; by interacting with collagen molecules they can promote network cross-linking and regulate collagen fibril formation. The fetal membranes are dominated by smaller proteoglycans such as decorin and biglycan, promoting reepithelialisation (Yeh, Chen et al. 2005). Decorin is present in the compact layer of the amnion and also in the mesoderm layer of the chorion. By binding to collagen type I and III, it enables lateral organization of the collagen fibrils and increases the tensile strength (Meinert, Eriksen et al. 2001; Meinert, Malmstrom et al. 2007).

The spongy layer at the interphase of the amnion and the chorion is dominated by the non-sulfated GAG named hyaluronan (Meinert, Eriksen et al. 2001), forming complex networks without attaching to a core protein. This increases the swelling pressure of the tissue by pressing the collagen fibers apart which may weaken the tissue. A gelatinous substance is produced and by this separating the amnion and the chorion. Thereby, it may decrease the tensile strength and increase the extensibility in amnion (Helmig, Oxlund et al. 1993; Arikat, Novince et al. 2006). This permits the amnion to slide on the underlying chorion minimizing the shear stress along the interface. An extraordinary (by three-fold) increase in hyaluronan and biglycan was observed in postlabour membranes by (Meinert, Malmstrom et al. 2007). Biglycan is preferentially localized in the vicinity of the cells, mainly located in the trophoblastic part of the chorion, and is thought to interact with type VI collagen. Further, it interferes with the decorin-collagen interaction and disrupts the structural integrity of the tissue (Meinert, Malmstrom et al. 2007).

Other adhesive glycoproteins

Fibronectin and laminin are other important components of the extracellular matrix of fetal membranes (Malak and Bell 1994; Hieber, Corcino et al. 1997; Bryant-Greenwood 1998).

Fibronectin are high molecular weight glycoproteins, organized into a fibrillar network contributing to the insoluble ECM. It is expressed by a wide variety of various cell types. Plasma fibronectin is synthesized in the liver and present in a soluble form in blood plasma,

being also important for thrombosis. Fibronectin have been linked to “glue” due to their multiple binding domains for cells as well as other matrix components, which stabilize the system of cells and matrix. This is accomplished by a specialized domain called RGD, corresponding to the amino acid sequence of Arginine (R), Glycine (G) and Asparagine (D), which mediates cell adhesion (Bryant-Greenwood 1998). By interaction with cell surface receptors, it regulates cell functions such as cell adhesion, migration, growth, and differentiation (R 1990).

Corresponding fetal membranes, immunolocalization studies have demonstrated a generalized distribution of fibronectin in the extracellular matrix, extending from the decidua to the amniotic basement membrane (Lockwood, Senyei et al. 1991). The most intensive staining was found in the chorionic cytotrophoblast. Disruption of such fibronectin binding, occurring before or at term, may result in the separation of the chorion from the decidua in the lower uterine segment.

Laminins are large heterotrimeric glycoproteins of three different polypeptide chains: α, β, and γ. Like fibronectin, laminins have multiple cell binding sites and interact with the extracellular matrix cells through cell surface receptors like heparin or integrin receptors (Lockwood, Senyei et al. 1991). Laminins also interact with collagen, heparin sulfate proteoglycans, and provide heparin-binding sites being critical for the basement membrane assembly (Li, Harrison et al. 2002); they anchor the cells to the basement membrane as well as connect the epithelial basement membrane to the underlying stroma through collagen IV. Therefore, laminins provide a significant strengthening function in the human amnion (Bryant-Greenwood 1998). Laminins undergo self-polymerization and form filaments and layered sheets, which initiate basement membrane assembly. When laminin polymerization is inhibited, basement membrane assembly seems to be disrupted even in the presence of other major constituents such as entactin, type IV collagen, and perlecan (Li, Harrison et al. 2002).

Other adhesive glycoproteins, including vitronectin, thrombospondin, tenascin, entactin (nidogen), nephronectin, and fibrinogen, are not discussed here.

1.3. Physiological functions of human fetal membranes

The fetal membranes, also referred to as the chorioamnionic membrane, create a highly specialized environment for the growing and moving fetus throughout gestation. Intact as well as healthy fetal membranes are required for an optimal pregnancy outcome throughout gestation.

The upright posture of the human pregnant female presents a far greater mechanical challenge for the fetal membranes compared to the ones of other species (Bryant-Greenwood 1998).

Fetal membranes filled with fluid are symbolically referred to "a balloon filled of water", inside which the fetus can move and float in the amniotic cavity. The amniotic fluid comprised in the fetal membrane sac increases gradually along the course of the pregnancy, reaching approximately 30 mL at ten weeks, 350 mL at 20 weeks, and 700 to 1000 mL at full term (Keith L. Moore 2008). These fluids together with the fetal membranes provide many functions for the fetus:

First of all, the environment protects the fetus from direct trauma by absorbing any impacts the mother may receive. Human chorioamnionic membranes must retain the full strength of their tensile properties to resist intra-amniotic fluid pressure. They need to tolerate increasingly vigorous fetal movements (Patrick, Campbell et al. 1982). Fetal membranes have to exhibit sufficient strength, but also extensibility in order to withstand stretching to approximately double their size by term (Parry-Jones and Priya 1976; Alger and Pupkin 1986). The amnion represents the essential part of the fetal membrane strength (Polishuk, Kohane et al. 1962; Oxlund, Helmig et al. 1990).

Secondly, the amniotic sac allows free fetal movement and permits musculoskeletal development; it facilitates symmetric growth and development.

Thirdly, the amnion serves as a barrier to ascending infections from the urogenital tract of the mother during pregnancy and thus, structural integrity is essential.

Additionally, the environment prevents the loss of heat, maintains a relative constant fetal body temperature as well as the amniotic fluid pH.

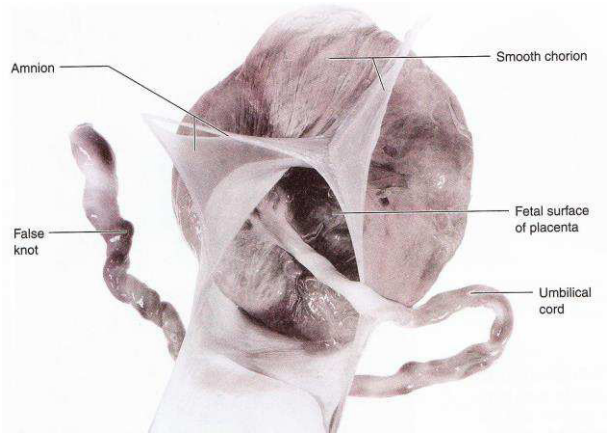

Figure 1.9 Placenta with the umbilical cord, amnion and chorion membrane after delivery (Keith L. Moore 2008).

Furthermore, the amnion (above all the epithelial cell layer) produces amniotic fluid which serves as source of oral fluid for the fetus. Substances such as nutrients, water, oxygen, electrolytes and hormones can reach the fetus by passive diffusion. Both layers, amnion and chorion, secret as well as absorb substances into the amniotic fluid and towards the uterus. By this, electrolyte and fluid homeostasis as

well as their exchange between the fetus and the mother via the membrane is reached to protect the embryo from drying out. The amniotic fluid turnover every 3 hours is maintained by large volume entering through the chorioamnionic membrane into maternal tissue fluid (Schmidt 1992). The floating fetus eliminates the amniotic fluid through the lungs and the epidermis, but produces fluid by the kidney (urine).

Finally, amnion is thought to play a role in the onset of labour, including the initiation and maintenance of uterine contractions (Lei, Furth et al. 1996). At term of the pregnancy under normal parturition, the chorioamnionic membranes rupture during labour and get ejected together with the placenta and the fetus from the uterus. An example of the placenta, the umbilical cord and the fetal membranes after delivery is presented in figure 1.9.

1.4. Rupture of human fetal membranes

Tissue failure as when the fetal membranes rupture is a normal action in human physiology, whereas other tissue failures (e.g. tendon/ligament failures, bone fractures, skin tears) are pathological processes. The mechanical rupture of fetal membranes is one essential part in the natural sequence of term delivery, but it can create serious complications if the membrane rupture occurs prior to term.

The natural rupture of the membrane (ROM) was traditionally thought to happen during parturition after the onset of uterine contractions and after opening of the cervix. However, the membranes failure is a direct consequence of a planned, biochemically mediated weakening process (Parry and Strauss 1998; Moore, Mansour et al. 2006) that may act in combination with physical forces (Lavery, Miller et al. 1982). At term, referring to completed 37 weeks of gestation, the fetal membranes' integrity and compliance get reduced, becoming more susceptible to rupture which may contribute to the initiation of parturition. A cascade of events has been implied to play a role in the initiation of rupture of the membranes (Osman, Young et al. 2006). This includes programmed cell death as well as the activation of catabolic enzymes such as collagenases which disrupt the extracellular matrix resulting in ruptured membranes. During or just prior to labour, MMP's and their inhibitors (TIMP's) regulate the breakdown of fetal membrane proteins.

Under normal parturition, labour starts at 38 - 42 weeks of gestation in the presence of intact fetal membranes. The physiological rupture of the membrane is associated with the birth process, frequently signalling the first indication of the delivery. However, in at least 10 % of

pregnant women at term, contractions follow the rupture of membranes. This situation is called “premature rupture of the membrane (PROM)”.

More significantly, in pregnancies delivered preterm (< 37 weeks of gestation), approximately one third experience ruptured membranes prior to the onset of contractions (Mercer, Goldenberg et al. 1999). This indicates that concomitant factors, e.g. biochemical processes, affect this event. In the case when fetal membrane rupture occurs before term and in absence of labour, it is defined as “preterm premature rupture of membranes (PPROM)”.

1.4.1. Preterm Premature Rupture of Membranes (PPROM)

Preterm premature rupture of the membranes (preterm PROM or PPROM) complicates approximately 3 % of all pregnancies and 30 - 40 % of all preterm deliveries (Mercer 2003). PPROM is far more difficult to manage than PROM happening at the end of gestation; it is a significant contributor to preterm birth and is a significant cause of gestational age-dependent neonatal morbidity and mortality. PPROM results in neonatal complications that vary in severity with the gestational age at which rupture and delivery occur. Membrane rupture before term with leakage of amniotic fluid through the cervix and vagina to the exterior leads to oligohydramnios (reduced amniotic fluid volume). The loss of amniotic fluid removes the major protection that the fetus holds against infection. Thus, oligohydramnios following PPROM is associated with increased risk for intrauterine infection and cord compression (Vintzileos, Campbell et al. 1985; Keirse MJNC 1989). In case of umbilical cord compression, the supply of nutrients and oxygen can be reduced. In addition, insufficient fluid in the fetal sac may interfere with normal lung development of a small fetus (Hofmeyr, Essilfie-Appiah et al. 2011). PPROM may therefore cause various fetal anomalies. Among surviving infants, common complications are pulmonary hypoplasia with respiratory distress syndrome, neonatal infection, and intra-ventricular haemorrhage, for instance (Rotschild, Ling et al. 1990; Vergani, Patane et al. 2000; Winn, Chen et al. 2000; Dewan and Morris 2001).

Overall, fetal survival after PPROM before 23 or 26 weeks of gestation is reported to be approximately 20 % (Dewan and Morris 2001) or 35-50 % (Bengtson, VanMarter et al. 1989; Spitz, Vossen et al. 1999), respectively. In general, prognosis is good after 32 weeks’ gestation (Jain and Sciscione 2011).

PPROM occurs after activation of a multifaceted and multistep pathway. Multiple epidemiological and clinical factors are considered to be promoters of PPROM. Studies about causative factors of PPROM are described below.

Studies of prematurely ruptured fetal sacs have pointed out the thinning at the fetal membranes rupture site, but their possibly reduced strength shall not be the explanation for its rupture (Artal, Sokol et al. 1976). Interestingly, amnions from pregnancies with prematurly ruptured membranes had even higher tensile strengths than those with timely membrane rupture (Lavery, Miller et al. 1980). Thus, it is not the general membrane strength that is important for rupture, but rather a local alteration that occurs near the site of the membranes overlying the cervix (Malak and Bell 1994; Malak, Sizmur et al. 1996). In a series of publications, it was hypothesized that human fetal membranes overlying the cervical opening of the uterus, also referred to the "zone of altered morphology", is more susceptible to rupture relative to the other areas of the same fetal membranes (Moore, Redline et al. 2009). The region at rupture was identified by disrupted collagen fibrils in different layers of the fetal membranes (Malak and Bell 1994). This zone was further characterized by a reduced thickness as well as increased collagen remodelling and apoptosis (McLaren, Malak et al. 1999; McParland, Taylor et al. 2003; El Khwad, Stetzer et al. 2005). These regional characteristics arise prior to the onset of contractions and continue until delivery (Moore, Mansour et al. 2006). Thus, it has been suggested that this weak zone is the fetal membrane's rupture initiation site (El Khwad, Pandey et al. 2006).

Aplin et al. observed interstitial collagen as well as other matrix molecule synthesis and deposition until term in cell and organ culture techniques, depending on the presence of vitamin C (Aplin, Campbell et al. 1986). Vitamin C as an antioxidant maintains the triple helical collagen chains in the reduced form which is preferred for maturation of the collagen fibers and thereby improves the stabilization. On the other hand, the collagen content in amniotic membranes was found to decrease significantly during the last 8 weeks of pregnancy; it may be reduced in membranes from prematurely ruptured fetal sacs (Skinner, Campos et al. 1981). According to Teodoro et al., term membranes of patients with premature rupture have 44 % less collagen than normal (Teodoro, Andreucci et al. 1990). Other researchers found no decrease but rather dissolution of fibers near the site of membrane rupture (Al-Zaid, Bou-Resli et al. 1980). Kanayama et al. analysed the collagen isoforms of normal and prematurely ruptured membranes (Kanayama, Terao et al. 1985). Altered ratios of type I, III and V collagens were recognized in specimens from prematurely ruptured cases. A

particular reduction of collagen type III was reported (Kanayama, Terao et al. 1985). There are lots of inconsistencies in the literature. Nevertheless, the crucial point is not the overall content of collagen, but rather the distribution of collagen isoforms, their cross-linkage being critical for the tensile strength and extensibility of fetal membranes, as well as the availability and activity of the respective cleaving enzymes. Thus, a structural defect of the ECM, particularly of the collagen fiber matrix, or an abnormal metabolism leads to the risk of PPROM. In the case a fetus develops the Ehlers-Danlos syndrome, an inherited connective disorder affecting the collagen synthesis, a high risk for PPROM exists.

The availability of copper ions is required to cross-link collagen fibers since copper is a cofactor for the enzyme lysil-oxidase mediating crosslinking. King et al. have shown that amniotic fluid levels of this Cu^{2+}-binding peptide are decreased in smokers. These data suggest a reduced availability of copper for cross-linking of collagens and may help explain the increased incidence of premature rupture of membranes in smokers. Furthermore, Shimizu et al. found inhibition of fibronectin expression in smokers. They explain the obvious correlation between smoking and preterm membrane rupture by the importance of fibronectins for cross-connecting collagen filaments and thus for the tensile properties of connective tissues.

The tightly regulated activity of MMPs and TIMPs is essential for the ECM remodelling and plays an important role in fetal membrane rupture. During normal parturition and also in case of preterm rupture of the membranes, increased levels of MMPs, particularly of MMP-9, were determined in the amniotic fluid. Furthermore, raised levels of TIMP-1 are observed in case of preterm rupture (Vadillo-Ortega, Hernandez et al. 1996). MMP-1 and MMP-8 disrupt the triple helical domain of collagen type I and III. Degradation is carried out by MMP-2 and MMP-9, which break down collagen typ IV, fibronectin and other proteoglycans (Vadillo-Ortega, Hernandez et al. 1996; Vettraino, Roby et al. 1996). An imbalance of MMP- and TIMP-activity and the related modifications in terms of the collagen structure can cause PPROM (Vadillo-Ortega, Gonzalez-Avila et al. 1990; Vadillo-Ortega, Gonzalez-Avila et al. 1995). Draper et al. 1995 found predominantly increased levels of MMP-9 beside other MMPs in membranes of women with preterm rupture (Draper, McGregor et al. 1995). In addition, Menon and Fortunato have demonstrated in 2007 that programmed cell death or apoptosis plays a substantial role in PPROM (Menon and Fortunato 2007).

Finally, it is well known that preterm rupture of fetal membranes occurs due to infections, e.g. chorioamnionitis (Naeye 1982), causing weakening of the membranes and/or induction of contractions (Wang, Parry et al. 2004).
Multiple risk factors and their interactions are thought to induce or cause the transition from uterine quiescence toward preterm labour or PPROM. Although many theories have been provided, the exact mechanism of provoking PROM and PPROM are still not clear. Additional research is crucial to clearly define the mechanisms and risk factors leading to PPROM. Understanding these mechanisms should allow clinicians to design appropriate interventions to reduce the incidence of preterm birth, fetal morbidity and mortality.
Two types of PPROM i.e. spontaneous (sPPROM) and iatrogenic (iPPROM) are distinguished in table 1.1 and described in the following sections.

1.4.2. Spontaneous PPROM (sPPROM)

Spontaneous preterm premature rupture of the fetal membranes (sPPROM) at less than 37 completed gestational weeks occurs in 1 - 2 % of all pregnancies and accounts for 25 - 30 % of all preterm deliveries (Parry and Strauss 1998; Simhan and Canavan 2005; Goldenberg, Culhane et al. 2008). Spontaneous preterm birth happens after spontaneous preterm labour and PPROM, called sPPROM (Goldenberg, Culhane et al. 2008). According to Goldenberg et al., it starts at least one hour before the onset of the contractions of the uterus. Spontaneous PPROM is a syndrome resulting from multiple causes, including infection or inflammation, vascular disease, placental abruption, preeclampsia, maternal stress, uterine over-distension (e.g. in twin pregnancy) as well as previously described antecedent biochemical remodelling (Parry and Strauss 1998; Bryant-Greenwood and Millar 2000). Risk factors for spontaneous preterm births include previous preterm birth, black race, periodontal disease, and low maternal body-mass index. The strongest predictors of spontaneous preterm birth are considered a short cervical length and an increased cervical-vaginal fetal fibronectin concentration (Goldenberg, Culhane et al. 2008).

Characteristic	Spontaneous PPROM (sPPROM)	Iatrogenic PPROM (iPPROM)
Cause	Multifactorial, often infectious	Invasive intrauterine procedure (amniocentesis, fetoscopy)
Defect size	Large (few centimeters)	Depends on size of instruments used (mm size)
Defect type	Often poorly delineated defect	Round wound with sharp demarcation
Defect location	Over cervical ostium (in 90 %)	At insertion site (often anterior or fundal)
Spontaneous resealing	Infrequent	Frequent
Occurrence in gestation	More common at advanced gestational age (usually 3rd trimester)	Soon after invasive procedure (often 2nd trimester)
Incidence	1 - 4 %	1 - 2 % after amniocentesis; 5 - 100 % after fetoscopy

Table 1.1 Major characteristics of spontaneous versus iatrogenic PPROM (Adapted from (Devlieger, Millar et al. 2006)).

In general, during spontaneous rupture of the fetal membranes, the generated defect is characterized by an irregular shape and poor demarcation, a large wound which is difficult to localize. In 90 % of the cases, spontaneous fetal membrane wounds are located in the cervical region of the uterus (Devlieger, Millar et al. 2006), see also table 1.1.

Spontaneous preterm birth was claimed to be an unusual and even unknown problem in most species, other than humans (Bryant-Greenwood and Millar 2000). The reason is unclear but may be due to our bipedal upright posture, imposing a greater mechanical challenge on the tissues than in other species.

1.4.3. Iatrogenic PPROM (iPPROM)

"Iatrogenic" preterm premature rupture of membranes (iPPROM) is described as PPROM resulting from invasive intrauterine procedures such as amniocentesis or fetoscopy. Increasing numbers of invasive procedures for diagnostic and therapeutic purposes are reported: The advanced maternal age does increase the demand for genetic amniocentesis, but also the number of operative fetoscopic procedures is constantly growing (Lewi, Van Schoubroeck et al. 2004).

Invasive procedures are performed using sharp needles or fetoscopes to enter the intrauterine cavity in order to evaluate or treat the fetus during pregnancy, as illustrated in an animal model in figure 1.10. Such interventions disrupt the intact fetal sac. After withdrawal of the needle or trocar, a surgical defect remains that may lead to temporary or permanent amniotic fluid leakage. Amniotic fluid leakage in the immediate postoperative period probably occurs more often than previously thought. More serious complications associated with fetal surgery

are separation of the fetal membranes from the uterine wall. The most relevant side effect in operative fetoscopy is the high risk for iPPROM that varies from 5 – 100 % (Harrison, Keller et al. 2003) and largely depends on the performed procedure and its complexity, the diameter of the used needle or fetoscope, the number of used ports and also the number of entries through the port. From previous studies, the numbers of incidence are given as following: 27 % (range from 11 – 53 %) after laser coagulation for twin-to-twin transfusion syndrome, 31 % (0 – 50 %) after shunt placement for lower urinary tract occlusion, and 26 % (0 – 100 %) for the treatment in twin pregnancies complicated by twin-reversed arterial perfusion (Ville, Hecher et al. 1998).

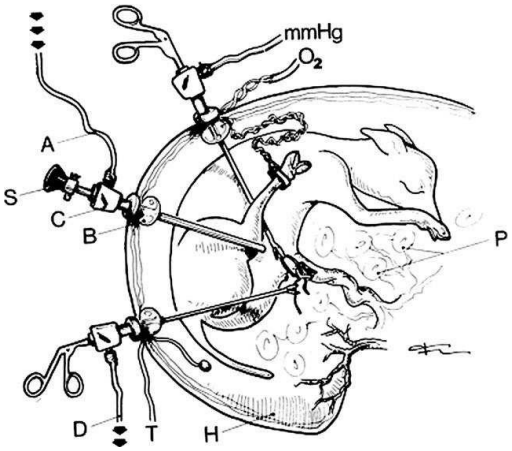

Figure 1.10 Ovine model for endoscopic fetal surgery (Deprest, Munro et al. 1995).

Since most invasive procedures are performed in the second trimester of pregnancy, iPPROM usually occurs at an early gestational age, with substantial risks for the fetus (perinatal mortality and morbidity) and the mother (chorioamnionitis) (Cox, Williams et al. 1988) and this may compromise the benefit of the intervention. The high risk for iPPROM after such invasive procedures strongly limits the clinical use of the fetoscopic technique and is a major obstacle for further development; therefore it is called the "Achilles heel" of fetal surgery (Deprest, Evrard et al. 1996). The serious complications highlight the need for further research to develop strategies and regimens to seal or heal defects in the fetal membranes after iPPROM. iPPROM will remain an inevitable complication and in order to rescue the pregnancy following iPPROM, an immediate sealing of the insertion port to prevent amniotic fluid leakage and reduce the undesired side effects is essential.

Defects after intrauterine interventions are characterized as described in table 1.1 by punctured wounds with sharp demarcation at the site of the intervention, revealing a minimal risk for infections.

1.4.4. Spontaneous healing of fetal membranes

Although fetoscopic interventions create acute wounds, the wound healing capacity of fetal membranes is very slow, if not absent (Benirschke 1995; Devlieger, Gratacos et al. 2000; Gratacos, Sanin-Blair et al. 2006; Liekens, Lewi et al. 2008). Human fetal membranes are not innervated and only poorly vascularised (Bourne 1962). Thus, the formation of a blood coagulum and epithelial wound healing, as described for the skin, does not occur. In a histological follow-up study of fetoscopic interventions performed during the second trimester, persistence of defects in the fetal membranes was observed several months later (Gratacos, Sanin-Blair et al. 2006). However, in most cases of post-amniocentesis amniorrhexis is limited, spontaneous resolution, and favourable pregnancy outcome is obtained. The defect might be concealed due to the attachment of the membranes to the decidua, the membranes could reseal through retraction or sliding over each other, or due to contractions and scarring in the myometrial and decidual layers of the uterus, rather than involving active healing mechanism at the level of the fetal membranes (Behzad, Dickinson et al. 1994; Gratacos, Sanin-Blair et al. 2006). Spontaneous resealing of the membranes might occur occasionally, suggesting that the fetal membranes, under certain conditions, have the capacity to heal or seal spontaneously (Phupong and Ultchaswadi 2006).

1.5. Current management of PPROM

Optimally, PPROM could be avoided through early identification of those at risk followed by effective interventions. However, as most women who develop PPROM have no identifiable risk factors, current management remains focused on interventions to optimize outcomes once PPROM is diagnosed.

Management of women being adversely affected by PPROM require an accurate diagnosis in addition to an individual assessment of the benefits and risks of continuing pregnancy versus expedited delivery. This evaluation requires an understanding of gestational age specific risks of neonatal morbidity and mortality and the potential benefits and risks of conservative management.

The most likely outcome for any patients suffering from PPROM in the absence of associated treatments, regardless of obstetric management or clinical representation, is birth within one week. In general, the earlier in gestation PPROM occurs, the longer is the latency period (the time between the rupture and the time of onset of labour). With expectant management (bed rest to enhance re-accumulation of amniotic fluid, to improve utero-placental perfusion and

thereby fetal growth), 2.8 - 13 % of women can anticipate cessation of fluid leakage and possible restoration of normal amniotic fluid volume (Johnson, Egerman et al. 1990). Since it is not possible to accurately predict which pregnancies will develop complications, such as infection or placental abruption, expectant management should be undertaken in the hospital.
The management of PPROM differs from institutions to institutions and includes immediate delivery or termination of pregnancy versus conservative management with or without the use of tocolytics and steroids in various combinations (Louis-Sylvestre, Morice et al. 1997).
In the case PPROM occurs with less than 24 weeks of gestation, and thus before potential neonatal viability, women should be counselled regarding the impact of immediate delivery and the potential risks and benefits of expectant management. If expectant management is followed, strict bed and pelvic rest to enhance the opportunity for resealing, as well as early identification of infection and placental abruption is essential. Otherwise, labour is induced. Between 24 – 31 weeks, expectant management, antibiotics (to reduce infectious and prolong the latency period), group B streptococcal prophylaxes as well as corticosteroids (to reduce the risks of RDS, perinatal mortality, and other morbidities) are recommended. The same procedure is advised after PPROM between 32 – 33 weeks, unless the corticosteroid use is managed differential. From 34 weeks onwards, streptococcal B prophylaxis is advised and delivery is targeted usually by induction of labour.

1.6. Novel treatment regimen for PPROM

"Clinically, attempts of plugging fetal membranes after established rupture as well as of preventive plugging of fetoscopic access sites have been undertaken", as previously reviewed (Devlieger, Millar et al. 2006; Zisch and Zimmermann 2008). Generally, the treatment of fetal membrane defects is complicated by several factors: the difficult accessibility, especially in the case of sPPROM, the thinness of the membranes, the deflection of the membranes from the uterine wall, and the wet application conditions. Nonetheless, several attempts to develop sealing or plugging strategies for fetal membrane defects have been evaluated in preclinical and clinical trials. Until now, the success of clinical trials was limited, the interpretation of the outcome due to low numbers was difficult and therefore none of these techniques has been introduced into clinical practice (Devlieger, Gratacos et al. 2000).

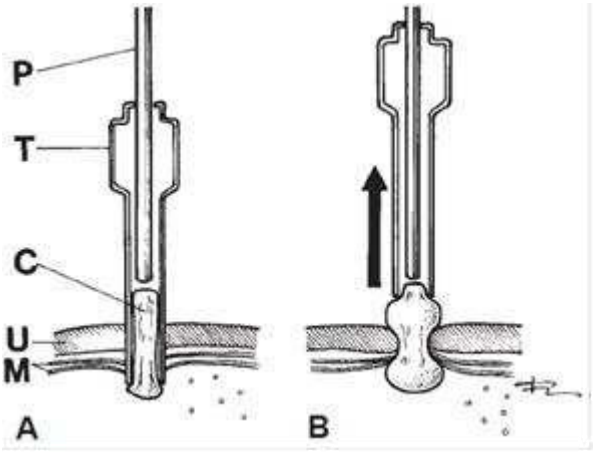

Figure 1.11 Technique of gelatine sponge plug insertion. A) Plug (C) is inserted in endoscopic cannula (T) and maintained in place with plunger (P) at level of uterine myometrium (U) and membranes (M). B) Cannula is withdrawn (arrow) (Luks, Deprest et al. 1999).

Investigations have been focussed on prophylactic measures prior to rupture rather than on therapeutic strategies after rupture of fetal membranes. Prophylactic plugging methods such as dry collagen or gelatine plugs have been clinically deployed under fetoscopic guidance to the defect before the fetoscope is finally retrieved (figure. 1.11). There is a need for such barriers or sealants for fetal membranes to close iatrogenic defects before rupture in order to prevent iPPROM. iPPROM is considered the prototype model to develop repair strategies, since defects in fetal membranes are clearly defined. Membrane sealing or plugging may not be appropriate for sPPROM, as membranes have undergone extensive extracellular remodelling and cellular apoptosis that may not readily be reversed. Additionally, repair of fetal membranes in the case of sPPROM will be difficult as it denotes large undefined wounds which are difficult to localize and with a high risk for infections. Thus, sPPROM repair poses a greater challenge with respect to iPPROM. The following chapters summarize different plugging and sealing methods which have shown potential for fetoscopic access sites after intrauterine interventions.

1.6.1. Plugging of fetal membranes

Candidate materials for plugging the fetal membranes are first of all dry and solid plugs or scaffolds (e.g. collagen, gelatine) that swell through fluid absorption and thereby self-lock into the lesion.

Chang et al. reported in 2006 about a cohort study including 27 patients, who were treated using the gelatine plug (Gelfoam) upon port retrieval in endoscopic surgery (Chang, Tracy et

al. 2006). In most cases, the gelatine plug was recognized by ultrasound for 2 - 3 weeks; thereafter it could not any longer be detected. A significantly lower PROM rate was reported with the use of the gelatine plug to seal the membranes, however, this may also be due to the minimized uterine and membrane trauma by using a new insertion technique.

Gratacos successfully sealed fetoscopic access sites in a rabbit model using collagen plugs (Gratacos, Wu et al. 2000). In combination with suturing the myometrial layer, this method resulted in functional restoration of the membrane integrity with preservation of the amniotic fluid and normal fetal lung to body weight ratio at term in 82 % of cases. Entrapment of membranes between the plug and the myometrium were revealed in histological evaluation but no anatomic repair of the membranes could be observed. In a case report of Quintero et al. the surgical repair of sPPROM at 17 weeks of gestation was presented using the collagen graft – the so-called amniograft – which was fixed to the membrane using fibrin glue (Quintero, Morales et al. 2002). Although they were unable to seal the membranes for the duration of the pregnancy in this case, the 2 - week span of complete cessation of amniotic fluid leakage after surgery represents an important step in the efforts to find better alternatives for patients with previable spontaneous rupture of the membranes. “Collagen fleece plugs (Lyostipt) are routinely used for prophylactic plugging of iatrogenic membrane defects following fetoscopic endoluminal tracheal occlusion for in utero therapy of congenital diaphragmatic hernia” (Bilic, Brubaker et al. 2010).

Other prophylactic plugging techniques such as scaffold-type plugs manufactured directly from decellularized amnion tissue have so far only been evaluated in animal models (Mallik, Fichter et al. 2007; Ochsenbein-Kolble, Jani et al. 2007). Such a decellularized amnion plug (DAM) can be observed in figure 1.12 which was applied on the rabbit uterus. Introduction and delivery of a fetal membrane patch through a narrow operative cannula to the site of the defect is sophisticated. After the plug has been deployed, the challenge of fixation to the membranes and the uterine wall remains as of the dynamic nature of the amniotic fluid and the uterine musculature.

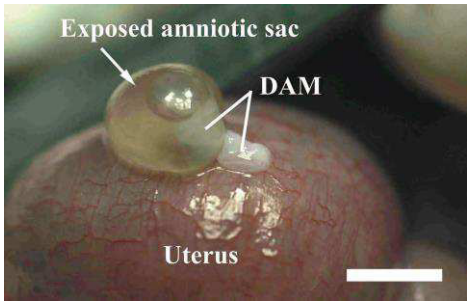

Figure 1.12 Decellularized term human amnion membrane plugs for closure of fetoscopic entry wounds in the exposed rabbit amniotic sac. The micrograph of the surgery site shows the dimension of a decellularized term human amnion membrane plug (DAM) spanning through the fetal membranes into the bulged amniotic sac.
Bar size: 1 cm (Mallik, Fichter et al. 2007).

Stable integration and low resorption of plugs grafted in fetal membrane defects are key requirements for long-lasting closure, especially due to the fact that fetal membranes exhibit low or even no wound healing capacity (Gratacos, Sanin-Blair et al. 2006) complicating the engraftment of plugs to defective membranes. Despite the potential feasibility of such plugging approaches to secure small fetal membrane defects, efficacy and safety need to be further investigated before translation to clinical practice. So far, most trials have been preclinical and were performed in the standard animal model, the mid-gestational rabbit model. But the experimental situation in rabbits is entirely different from the human clinical situation, and primarily suited to judge technical feasibility. Appropriate animal models for preclinical trials with plugging materials and plugging techniques have to be evaluated. However, the surgical plugging method is not intended, nor may be suitable for grafting large and sparsely defined defects that can be generated upon spontaneous PPROM.
Obviously, there is a need for new material strategies, which provide an immediate, non-toxic, and a physically stable barrier to the amniotic fluid with the characteristics of tissue-adhesiveness, long-term stability and engraftment under wet conditions.

1.6.2. Injectable sealants for fetal membrane repair

A promising alternative to plugging of fetal membranes exhibit prophylactic sealing by the deployment of gluing materials to fetoscopic access sites at the time of invasive intervention. Applied materials must be fetoscopically applicable, non-toxic, strongly bonding to tissues and immediately forming a watertight barrier that is physically stable to the amniotic fluid. Persistent key requirements are biocompatibility, slow resorption and long-term engraftment of tissue adhesives and scaffolds to minimize the injury and the risk for rupture. Thus, there is a great demand for new material innovations of fetoscopy-applicable sealing materials. Injectable surgical sealants such as self-cross-linking polymers (e.g. fibrin glue) represent a potential option. If such a material approach will be advantageous to seal fetal membranes by fetoscopic application is not yet determined.

Fibrin based approaches

Fibrin is a provisional wound healing matrix, which is established during the formation of the blood clot by thrombin mediated cleavage of fibrinogen (physical network) and subsequent cross-linking with factor XIII. Commercial products consisting of purified human fibrinogen and thrombin also named fibrin glue or fibrin sealant have been employed successfully as

tissue glues in various medical applications such as in the wound healing for years (Atrah 1994) and might thus have the potential to reseal fetal membrane defects. As a human derived product, it is known to be non-toxic, which makes it of great interest for a clinical approach. Fibrin glue promotes a sealing within seconds to minutes, depending on its composition.

Treatment of PPROM by the use of fibrin glue was first reported by Genz in 1979 (Genz 1979). They applied fibrin sealant as a cervical plug, which was meant to stop further amniorrhexis, to act as barrier between the uterus and the vagina, and to prevent ascending infections. Intracervical fibrin sealant was also applied by Sciscione et al. in 2001, which treated 12 women with PPROM suffering of severe oligohydramnios before 24 weeks of gestation. Seven neonatal survivors resulted from the 12 pregnancies but six of them had complications due to prematurity (Sciscione, Manley et al. 2001). Fibrin tissue sealants increased the post-rupture latency period and the neonatal survival rate, but it remains unclear whether closure of the membranes has been obtained.

In 1980, Diaz-Zagoya et al. reported on in vitro sealing of ruptured fetal membranes with fibrin cryoprecipitate (Diaz-Zagoya 1980). The sealed membranes were able to tolerate hydrostatic pressures up to 60 mmHg without rupture. They concluded that fibrin seals ruptured membranes in vitro. Harmanli et al. assessed the tensile strength characteristics in a vertical test system of artificially punctured membranes (n = 30) after treatment with fibrin glue (Harmanli, Wapner et al. 1998). Effective improvement of the structural integrity of the human fetal membranes by fibrin glue was reported. Nevertheless, fibrin glue was degraded within days to weeks (Harmanli, Wapner et al. 1998). Papadopulos et al., showed a lack of efficacy by using fibrin sealant in the mid-gestational rabbit model (Papadopulos, Van Ballaer et al. 1998). Due to the fact of rapid resorption, the usefulness of fibrin-based sealants is limited (Mallik, Fichter et al. 2007). Small number of treated cases and the absence of control groups complicate final conclusions about fibrin glue.

In one single case, fresh maternal blood was deployed under ultrasonic guidance to form a clot patch at 16 weeks of gestation for sealing of a membrane defect induced by amniocentesis (Sener, Ozalp et al. 1997). Although amniotic fluid leakage stopped within 12 h and re-accumulation of amniotic fluid was observed, ultra-sonographic guidance investigation showed that the blood clot was gradually diminished and completely disappeared within three weeks. Nevertheless, after normal progression of pregnancy a healthy baby was born at term.

Maternal platelets along with fibrinogen (the so-called amniopatch) injection trans-abdominally into the amniotic cavity (Quintero, Romero et al. 1996; Quintero, Morales et al. 1999) were reported for the sealing after intrauterine interventions. The hypothesis was that platelets become activated and the fibrin clot formation would initiate healing. Although the sealing of the fetal membranes was achieved, the detailed mechanism by which the amniopatch successfully closed the defect is not understood, as the injection was performed into the amniotic cavity without knowing the exact location of the defect. It is uncertain how the material found the defect and could effectively seal it.

Table 1.2 lists different sealing strategies using fibrin-based approaches which were applied in clinics in order to prevent iPPROM.

In 2001, Quintero reported another two cases of iPPROM that were treated by the amniopatch technique after fetoscopy. Two of the six cases that were treated by the amniopatch were complicated by sudden intrauterine death. It has been hypothesized that hemodynamic changes beside the platelet activation could induce fetal demise. These serious complications require further experimental work on the mode of action and the exact nature as well as the quantity of blood products required to reduce fetal side effects (Lewi, Van Schoubroeck et al. 2004).

Study	n	GA @ PPROM (wk)	GA @ treatment (wk)	Intervention	Outcome	Complication
Sener et al (1997)	1	16.2	17.0	Extra-amniotic instillation of 4mL maternal blood	Spontaneous birth @ 37wk	-
Young et al (2000)	1	17.3	20.6	Fetoscopic application of maternal platelets & fibrinogen/thrombin	Delivery at 32.3 weeks	-
Quintero (2001)	22	15.6-24.0	18.7	Intra-amniotic Instillation of maternal platelets & cryoprecipitate	Membrane sealing in 10/22 cases; 18/32 survivors	1 survivor surgery for residual bands; 2 sudden intrauterine fetal deaths
O'Brien et al (2002)	1	17.0	19.0	Transabdominal instillation of gelatin sponge @ 19 & 21wk, McDonald cerclage @ 21wk	Delivery @ 36wk	Unilateral club foot
Young et al (2004)	3	15-23	16-24	Fetoscopic application of maternal platelets & fibrinogen/thrombin	Delivery of viable infants @ 26, 32 & 34wk; 1 *TOP* after unsuccessful procedure	-
Lewi et al (2004)	2	17.2 & 22.0	18.5 & 23.0	Intra-amniotic instillation of maternal platelets & cryoprecipitate	Uneventful neonatal outcome in the survivors	Chorioamnionitis
Cobo et al (2007)	5		16-24	Chorionic villus sampling & Amniopatch application	1 alive & well @ 26wk, 1 TOP, 3 IUFD	-
Pathak et al (2010)	3		18, 23 & 25	TTTS & Amniopatch application	Single or double recipient survival, alive & well	-

Table 1.2 Reports on fetal membrane sealing strategies after iatrogenic preterm PROM (Adapted from (Devlieger, Millar et al. 2006)). GA: Gestational Age (wk = weeks), TOP: Termination of pregnancy, IUFD: Intrauterine fetal death, TTTS: Twin-to-twin transfusion syndrome.

A similar technique was applied in four human cases by Young et al. using platelets, fibrin and powdered collagen slurry to close post-amniocentesis rupture sites (Young, Roman et al. 2004). Rapid sequential injection of the mixture was deployed at the site of the defect, and of the trocar placement. Three patients delivered viable infants at 26, 32 and 34 weeks of gestation and in one patient the membranes ruptured 12 h after the sealing procedure, so that for the termination of the pregnancy was agreed. This procedure was also performed after the

occurrence of sPPROM in four patients but with a very poor outcome. Thus, the authors have concluded that this technique shows no benefit for spontaneous ruptures and is only effective after amniocentesis (Young, Mackenzie et al. 2004).

Mussel Glue

Mussel glue tissue adhesive is a two-component system, providing a self-crosslinking polymer with the excellent property to form strong and long-lasting bonds. Mussel glue has been inspired by the composition of proteins that are secreted by marine mussels and allow these organisms to firmly adhere to almost any surface. The wet adherence of native mussel adhesive proteins is due to the unusual amino acid residue of hydroxylated tyrosine's (3,4-dihydroxyphenylalanine (DOPA)) that is present in high concentrations in the foot proteins of mussels (Waite 1999; Lee, Dellatore et al. 2007; Lee, Lee et al. 2007). The work in the group of P.B. Messersmith and other laboratories have demonstrated that the wet adherence ability of mussel foot proteins can be translated onto synthetic polymers, such as polymer ethylene glycol (PEG) as an inert and biocompatible macromolecular support, and by incorporation of DOPA or DOPA analogues, see figure 1.13 (Yamada, Chen et al. 2000; Lee, Dalsin et al. 2002; Burke, Ritter-Jones et al. 2007). Previous work has demonstrated that DOPA-functionalized polymer ethylene glycol (PEG) precursors cross-link through the addition of sodium periodate (a strong oxidative reagent) that mediates oxidation to form adhesive hydrogels with high robustness (Lee, Dalsin et al. 2002). A redox reaction between sodium periodate and the catechol side chain of DOPA gives rise to reactive quinone species which yield covalent bonds to amine residues in proteins as well as other quinones and thus lead to crosslinking reactions (Brubaker, Kissler et al. 2010). Rapidly, the polymer network with more than two DOPA molecules couple together. The catechol groups incorporated into synthetic polymers enhance the wet adhesive properties (Brubaker, Kissler et al. 2010). They are essential for the effect of water displacement in order to allow for glue interaction and adherence to any wet surface. These terminal DOPA-groups result in polymer network formation and rapid gelation due to oxidative polymerization (Lee, Dalsin et al. 2002).

The herein applied mussel glue polymer is a 4-arm branched polymer containing the simplified form of DOPA, a reactive catechol group, as modified end groups. This formulation possesses appealing characteristics for the use as fetal membrane sealant. Mussel glue presents very slow hydrolysis over several months in vivo, presenting a long term sealing, fast gelation time (< 1 minute) and remarkable tissue adhesion under semi-wet

conditions (Brubaker and Messersmith 2011). A recent investigation analysed the bonding properties of glues between porcine dermal tissues. Bonds generated by a mussel-mimetic adhesive performed five times as strong as those formed by fibrin glue (Burke, Ritter-Jones et al. 2007). The mean shear strength of PEG-DOPA on porcine skin was reported 35 kPa (SD=12.5 kPa, n=11). An additional advantage is that engineering mussel glue can yield a specific biological application without change of its chemical properties, but simply by the variation of the initial precursor concentration (DOPA), the architecture of the PEG backbone or the concentration of sodium periodate (Lee, Dalsin et al. 2002).

Bilic et al. evaluated liquid sealing materials, as an alternative for preventive plugging, in terms of their bonding capacity to semi-wet fetal membrane tissue as well as their toxicity in vitro. Mussel glue adhesive formed a continuous layer tightly bound to the tissue in contrast to four other sealants resulting in no, weak, or partial bonding or even the change of the overall membrane morphology (Bilic, Brubaker et al. 2010). Mussel glue showed comparable biocompatibility to fibrin glue and no significant cytotoxic effects were reported with respect to amnion cells in vitro.

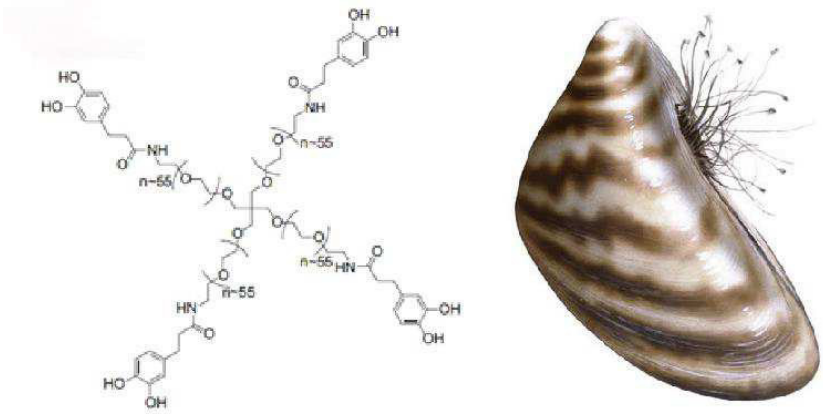

Figure 1.13 Structure of the polymeric component – PEG inclusive the terminal DOPA groups (Brubaker, Kissler et al. 2010)

A possible drawback might exhibit the use of the strong oxidative reagent called sodium periodate, used to initiate the polymerization, which is known to be strongly irritating. However, the contact of periodate with PEG does not only give rise to the cross-linking of the polymer but also results in reduction of periodate to less harmful oxidative species. Furthermore, in vivo implantation of mussel glue in C57BL6 mice elicited minimal acute or chronic inflammatory response and maintained an intact interface with the supporting tissue for up to one year (Brubaker, Kissler et al. 2010). Although exhibiting an expensive 2 -

component system, mussel glue sealant could be the ideal working matrix in such an approach, as it bonds tightly to the fetal membrane tissue. Nevertheless, the full effects of mussel glue on uterine contractions, fetal survival and integrity will require further evaluation.

Other tissue sealant applications

Previous studies have suggested tissue adhesives other than fibrin and mussel glue to perform the closure of fetal membranes after open intrauterine surgery or fetoscopy. Although cyanoacrylates can lead to inflammation and tissue necrosis, thrombotic events, calcifications, and release of formaldehyde to the tissue, it has been proposed as a candidate sealant due to its extreme adhesion capacity to biological tissue (Basaran, Vural et al. 2009). Furthermore, cyanoacrylates are difficult to handle and thus the application into the intrauterine cavity to seal fetal membrane defects can be very challenging.

Cortes et al. proposed a pre-emptive membrane sealant technique in which model a so-called pre-sealant is placed before membrane puncture and then used as a ‘‘working’’ matrix through which puncture or trocar manipulation would proceed. The matrix supports the fetal membranes and prevents an increase in the defect size throughout the invasive procedure. They suggest that the clinical application of this method potentially may eliminate the risk of iatrogenic PPROM (Cortes, Wagner et al. 2005). In this study, BioGlue and CoSeal demonstrated an effective sealing performance at membrane pressures that correspond to physiologic intrauterine pressures. BioGlue (composed of purified bovine serum albumin and glutaraldehyde), however, did not qualify as an adequate candidate as a ‘‘working’’ matrix for the pre-sealant method. Limitations may include the risk of in utero exposure to glutaraldehyde and potential infectious risk of bovine derived albumin. CoSeal, on the other hand, demonstrated many favourable properties such as effective sealing and clinically useful qualities. Disadvantages include the 2 - step preparation, the high cost of the glue, and the potential for a moderate inflammatory tissue response.

Therefore, the development of a reliable membrane sealant technique may go a long way in enabling the continued development of fetal surgery. The future success of the fetal membrane sealing will rest on the development of a specifically designed tissue sealant.

1.7. Mechanics of the fetal membranes

The thin and soft fetal membranes are constantly subjected to applied stresses and must support the bulk loads of the fetus and the amniotic fluid as well as tolerate local deformation associated with fetal movement during the entire gestation (Oyen, Cook et al. 2004).
Ultimately, the failure process of fetal membranes is intrinsically mechanical, nevertheless the course of biological and mechanical events associated with PPROM are not very well understood (Polzin and Brady 1991).
In order to develop the basis for successful treatment and prevention of premature fetal membrane failure, apart from conservative management such as membrane glues or patches, it is necessary to know the fetal membrane's structural and biomechanical behaviour as well as the physiological requirements. Understanding the baseline mechanical response of the fetal membrane component layers, the amnion and the chorion as well as the membrane strength in more detail, is a necessary foundation to investigate on interventions targeted for restoring membrane function following early rupture (Oyen, Calvin et al. 2006). Therefore, it is important to identify the distribution, quantity and the degree of organization of potential strength bearing components such as the extracellular matrix proteins collagen and elastin.
Although amnion contributes little to the fetal membrane thickness, this layer is incredible stiff and strong but not very extensible containing dense collagen fibrils. Amnion accounts for the dominant part in the mechanical response of intact fetal membranes (Oyen, Cook et al. 2004). Previous studies suggest that amnion reflects a remarkably resilient tissue material (Oxlund, Helmig et al. 1990; Oyen, Calvin et al. 2006). Its mechanical behaviour is of a typical non-linear viscoelastic material, time and history dependant (Oyen, Cook et al. 2004). Collagens, presenting the load-bearing network structure of the amniotic tissue, undergo a constant turnover throughout pregnancy to accommodate the increasing volume and tension as gestation progresses (Kanayama, Terao et al. 1985).
Chua and Oyen have summarized the available mechanical data for key physical properties of the chorioamnion membrane. The failure strength was evaluated to be a consistent value of 0.9 MPa across many different studies and even different testing methodologies. Mahmood et al reported that a membrane with high elastin content but less collagen allows for large extensibility, whereas a membrane with more collagen and low amount of elastin provides for low resistance to deformation and tends to limit the possible elongation (Jabareen, Mallik et al. 2009; Buerzle, Haller et al. 2012). To obtain an accurate and detailed picture of the elastic properties of the membranes, further investigation and more data is needed (Chua and Oyen

2009). The fetal membranes stiffness and strength is not only determined by the quantity of ECM proteins such as elastin and collagen, but also by the extent of cross-linking of these molecules characterizing the strength-bearing network. The mechanical properties are related to the type & the arrangement of fibrous proteins in the connective tissue.

Gestational aging, inflammation and labour have been shown to decrease fetal membrane strength and elasticity (Oyen, Cook et al. 2004; Calvin and Oyen 2007). Furthermore, biochemical changes have been reported to occur in the region overlying the cervix and are associated with physical weakening of the fetal membranes (Malak and Bell 1994; McLaren, Taylor et al. 2000; El Khwad, Stetzer et al. 2005). This area, known as the "weak zone" or the "zone of altered morphology", is an area of decreased work to rupture, decreased stiffness, and decreased ductility at the time of rupture (Reti, Lappas et al. 2007; Joyce, Moore et al. 2009). In studies of fetal membranes after spontaneous rupture, this "weak zone" area is bisected by the rupture tear line (El Khwad, Pandey et al. 2006).

The physical properties of fetal membranes (strength, stiffness, etc.) vary significantly between samples and between different areas of the same membrane (Artal, Burgeson et al. 1979) and comply with the heterogeneity of their biochemical and histological characteristics (Moore, Mansour et al. 2006). The weak zone overlying the cervix at near term may intensify the inhomogeneous mechanical properties.

1.7.1. Biomechanical test methodologies

In vitro mechanical testing allows for controlled characterization of the deformation and failure behaviour of soft tissue materials. Biomechanical measurements, to gain knowledge about the physical properties of fetal membranes, e.g. viscoelastic behaviour and strength, were first conducted in 1863 by Poppel (Duncan 1867; Chua and Oyen 2009). Since that time, numerous investigators have performed in vitro biomechanical studies in order to better understand the development of fetal membrane failure. Three types of mechanical test techniques were utilized (see also figure 1.14):

(1) Uniaxial tensile testing: The membrane is placed between two grips and gradually pulled apart while the resulting forces and strains are monitored (Artal, Sokol et al. 1976; Artal, Burgeson et al. 1979; Oxlund, Helmig et al. 1990; Helmig, Oxlund et al. 1993; Oyen, Cook et al. 2004; Calvin and Oyen 2007) (Fig. 1.14, Left)

(2) Biaxial burst (inflation) testing: A section of the membrane is clamped in a circular ring and increasing pressure is applied, either via air or fluid. The height of the

membrane is monitored (Polishuk, Kohane et al. 1962; Maclachlan 1965; Parry-Jones and Priya 1976; Lavery and Miller 1977; Lavery and Miller 1979; Al-Zaid, Bou-Resli et al. 1980; Lavery, Miller et al. 1982; Schober, Kusy et al. 1994; Calvin and Oyen 2007). (Fig. 1.14, Middle)

(3) Biaxial puncture testing: A spherically tipped metal probe displaces the central portion of a membrane that is clamped in a ring, perpendicular to the plane of its surface. Resulting forces are measured (Lavery, Miller et al. 1982; Schober, Kusy et al. 1994; Pressman, Cavanaugh et al. 2002; El Khwad, Stetzer et al. 2005; Arikat, Novince et al. 2006; Moore, Mansour et al. 2006; Oyen, Calvin et al. 2006; Calvin and Oyen 2007). (Fig. 1.14, Right)

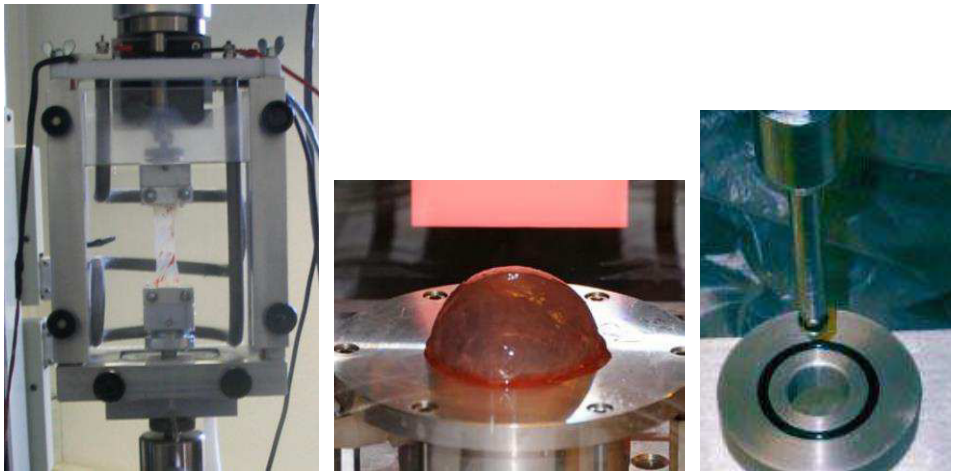

Figure 1.14 Left) Tensile test machine, Middle) Inflation Testing, Right) Puncture Testing

Of these methodologies, uniaxial tensile testing does not provide a physiological testing state as stresses are applied uniaxially, whereas the fetal membranes are physiologically loaded under a complex biaxial state. However, uniaxial tensile testing is used as it is simple to calculate the stress-strain relation.

Burst testing best mimics physiology as the deformation of the membrane most closely approximates the in vivo mechanical deformation, simulating the fluid pressure on the membrane over a dilated cervix. The disadvantage is that relatively large membrane pieces are required for this test method resulting in very few samples per placenta.

Therefore, puncture studies were initiated to solve the problems of burst testing requiring large tissue samples. Since every individual membrane is highly inhomogeneous, it is desirable to perform 10 - 20 tests per placenta which can only be achieved when smaller sample sizes are used. Puncture testing performs biaxial stress as well and has become

accepted since the work of Schober et al., who correlated the results of the physiological burst testing with puncture testing (Schober, Kusy et al. 1994). They demonstrated that the mechanical properties derived from the puncture testing method were equivalent to those obtained by the burst testing method, if the ratio of the tissue fragment to probe surface area was extrapolated. Thus, physical properties obtained by puncture testing on small samples can be mathematically related to burst testing results allowing multiple membrane samples to be tested from a single membrane (Moore, Mansour et al. 2006).

Important key mechanical terms are explained by short descriptions in the following paragraph:

- Force (F): The physical force carried out to the fetal membranes by an external source (e.g. a plunger)
- Stress (σ): The forces per original cross sectional area; $\sigma = F/\omega_0 t_0$, where ω_0 = initial width and t_0 = initial thickness
- Membrane tension (T): Alternative stress measure expressed as force per unit length (N/m); $T=F/\omega_0$, often applied if the thickness measurements are difficult and/or of low accuracy
- Stretch (λ): Measure of deformation defined as $\lambda = L/L_0$, where L is the current length of the fiber and L_0 is the original length.
- Strain (ε): Extension (displacement) of the sample per initial length; $\varepsilon = \delta/L_0$

Commonly reported mechanical properties are the elastic modulus (Young's modulus, E), to quantify the stiffness of the tissue, as well as the tensile strength (failure strength, σ_F), which is equal to the maximum stress level before failure. The parameter of interest to express the fetal membranes fracture behaviour is the fetal membranes strength. For a tensile test, the failure strength is simply the stress at maximum load $\sigma_F = F_{max}/\omega_0 t_0$. The elastic modulus, E, is the slope of the stress (σ) – strain (ε) curve leading to linearization the data for viscoelastic materials; since in soft tissues stress and strain typically are not linearly behaving, the tangent modulus is frequently reported. The higher the Young's modulus, the stiffer is the material.

In a biaxial inflation test, the burst pressure (P) is related to the failure strength of the membrane via the radius (r) of the "bubble" just before rupture and the sample thickness using the assumption of a hemispherical bubble: $\sigma_F = P_{max} r/2t_0$.

1.7.2. Studies on mechanical properties of fetal membranes

The comparison of the previously performed mechanical testing's to evaluate fetal membrane's properties is difficult due to inconsistencies in the results. In controlled mechanical tests it has been noted that amnion and chorion do not fracture simultaneously (Oxlund, Helmig et al. 1990; Oyen, Cook et al. 2004). Remarkably, the membrane component rupturing first during stress loading is not consistent between reports. Artal et al., Lavery and Miller, Oyen et al. as well as Moore et al. state that chorion ruptures first (Artal, Sokol et al. 1976; Lavery and Miller 1979; Oyen, Cook et al. 2004; Moore, Mansour et al. 2006), in contrast to Schober et al. and Helmig et al. reporting that amnion ruptures first (Helmig, Oxlund et al. 1993; Schober, Kusy et al. 1994). The group of Polishuk et al., Parry-Jones and Priya, Al-Zaid et al., Lavery and Miller as well as Moore et al. concluded that the amnion is stronger than chorion (Polishuk, Kohane et al. 1962; Parry-Jones and Priya 1976; Lavery and Miller 1979; Al-Zaid, Bou-Resli et al. 1980; Moore, Mansour et al. 2006). The discrepancy in the rupture sequence might be explained by different applied testing systems and modes, where different boundary conditions of test methods can reveal distinct results.

Only a few studies tried to relate the physical properties with biochemical features of the membranes. Comprehensive correlations to the histology are missing. In some studies, the thickness was not measured complicating the data comparison (Pressman, Cavanaugh et al. 2002; Arikat, Novince et al. 2006; Pandey, Jaremko et al. 2007). However, thickness measurements are essential to make accurate calculations of material properties (Calvin and Oyen 2007; Chua and Oyen 2009), as there are apparent relationships between membrane thickness and strength – an inflamed, hypertrophic membrane is unlikely to be as strong as one consisting of dense and organized collagen. Also Lavery and Miller have reported deformation, thinning of the fetal membranes and by this weakening of the tissue induced by mechanical stretch. Furthermore, Lavery and Miller demonstrated that fetal membranes exhibit viscoelastic properties of creep (increased deformation over time at constant load), stress relaxation (decreased load required over time to maintain a constant deformation) and plastic (non-recoverable) deformation (the thinned tissue did remain and not return in its original configuration upon removal of the load) (Lavery and Miller 1979).

A substantial part of the total work required to rupture the fetal membranes is needed to separate amnion and chorion. The intact membranes demonstrate a sparse interconnection between amnion and chorion (Fawthrop and Ockleford 1994), adding to the membrane strength, therefore, separation of the two layers leads to minimal tissue disruption. But it

remains unclear, whether shear forces are entirely responsible for the separation of amnion and chorion. It may be facilitated by biochemical changes during gestation such as described for the weak zone overlying the cervix. Strohl et al. have observed increased separation and decreased adherence of the membranes with increasing gestation as well as with labour. Thus, the layer separation was associated as a part of the fetal membrane weakening process during normal parturition (Strohl, Kumar et al. 2010).

Paradoxically, the rupture strength was demonstrated to increase after repeated stretching using 5 - 10 cycles (Lavery and Miller 1977; Lavery and Miller 1979; Lavery, Miller et al. 1982), rather than to decrease, whereas the work to rupture the membranes decreased. After many cycles, the force to rupture did increase. The same pattern was also observed for amnion tested separately: Rupture strength initially increased, called strain hardening, while the work to rupture decreased. Choriodecidua did not exhibit properties of stretch induced deformation (Moore, Mansour et al. 2006).

1.7.3. Further considerations regarding membrane modelling

Current membrane failure testing techniques provide limited information on fetal membrane tissue mechanics. In order to facilitate the data analysis, the target region of the test specimen should be exposed to uniform stress and strain fields. Moreover, the target sample region should be small as well as located away from the outer boundary of the test samples to avoid impacts from the specimen grips onto results (Joyce, Moore et al. 2009). Planar biaxial testing, as visualized in figure 1.15, can provide these necessary boundary conditions and is an approach to study sub-failure mechanical properties, a prerequisite for understanding fetal membrane failure. This approach was used by Joyce et al. in 2009. Their results demonstrate that the fetal membranes exhibit modest mechanical anisotropy, meaning that the tissue's response to stretch was different along the two axes, χ_1 and χ_2. These initial results suggest that the fetal membrane's collagen fibers become fully loaded and are straightened well below physiological loading levels. Moreover, this indicates that collagen fibers have very little structural reserve in comparison to other soft tissue (which are not aimed at failing), thus failure is probably easily facilitated during labour. By stretching collagen molecules in the loaded tissue, new access sites may open up, resulting in greater susceptibility to enzymatic degradation.

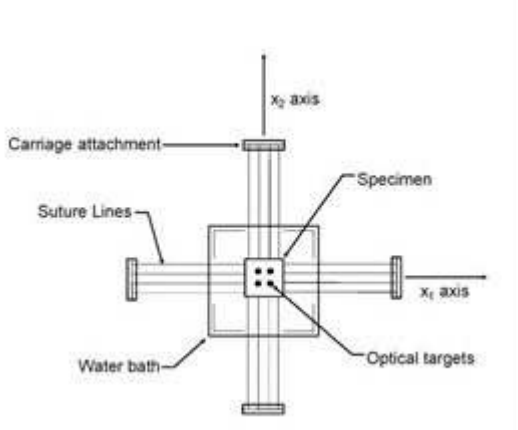

Figure 1.15 Planar biaxial device (Joyce, Moore et al. 2009).

Another approach to characterize the sub-failure response of fetal membranes is the use of constitutive models (i.e. stress-strain) that can help to elucidate the correlation between the structural components and the resulting mechanical behaviour of tissues.

Two scenarios of how collagen fibers behave during the tissue failure process were demonstrated by Joyce et al. according to figure 1.16. A single undulated collagen fiber is initially crimped and cannot bear load (figure 1.16a). By increasing the stretch λ, the fiber begins to straighten and bear load. At this point the fiber is considered to be linearly elastic with slope K, known as the elastic constant.

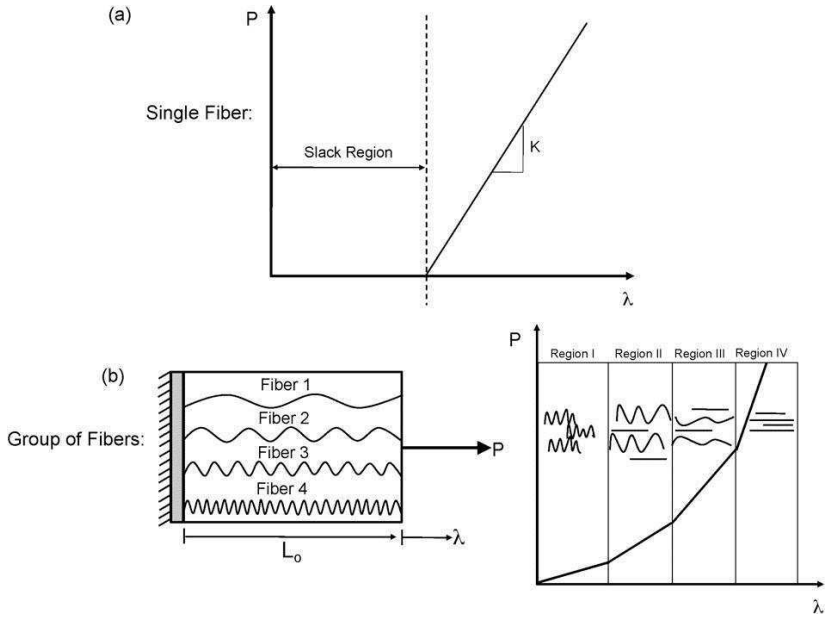

Figure 1.16 A) Single collagen fiber. Traditional recruitment modelling assumes that a collagen fiber does not bear load until it is fully straightened. B) Group of collagen fibers. Gradual recruitment of collagen fibers results in a non-linear stress-strain relationship (Regions I, II, III). Once all collagen fibers become straightened, the stress-stretch curve turns into a linear region (Region IV) (Joyce, Moore et al. 2009).

In figure 1.16b a group of collagen fibers is examined, each fiber presenting a different degree of undulation and thus being recruited at a different level of λ. Fiber one shows the least amount of crimping and therefore straightens first. With a higher λ also fiber two becomes straightened, followed by fiber three and then fiber four.

1.8. General scope of the thesis

IPPROM is still an unsolved problem in obstetrics leading to serious complications and called the “Achilles heel” of fetal surgery. Fetal membranes exhibit limited or even no healing capacity, thus there is a need to physically plug the fetoscopic access site to prevent amniotic fluid leakage. Despite various attempts to repair the fetal membranes after invasive procedures, no technique for prophylactic plugging has been implemented into clinics so far.

In order to advance in the fetal membrane sealing development to prevent iPPROM, it is necessary to meet the mechanical and normal physiologic requirements to succeed. Therefore, we need an appropriate in vitro model to simulate the relationship of the fetal membranes and the amniotic fluid environment as well as to evaluate potential sealing strategies. The following research has focused on a standardized biaxial inflation procedure in order to deepen our knowledge about the membranes’ physical properties, to analyse the related biochemical constituents as well as to investigate on a new candidate sealing material.

First of all, a novel custom-built membrane inflation device that permits ex-vivo physical testing of fetal membranes in a close to physiological way (water-filled 'bubble', radial stretch) was implemented. For this purpose, a standardized mounting procedure and a test protocol for the membrane inflation had to be developed and set up. The aim of the first study was to characterize the fetal membranes using the inflation experiment, in order to correlate the measured mechanical response with the tissue’s micro-constituents (elastin and collagen) of intact fetal membranes, but also of the amnion and chorion membranes solely. This allowed us to determine how the mechanical properties are affected by the biochemical material parameters. For the biochemical analysis, the hydroxyproline protocol had to be elaborated as a first step in order to determine the collagen content of the membrane tissue. Additionally, the established histological method of Mallik et al. that permits the exact determination of thickness of amnion and chorion was applied (Mallik, Fichter et al. 2007).

In the second study, the deployment and barrier properties of the new injectible mussel glue sealant were investigated for the closure of fetoscopic punctures using standardized elastomeric membranes in the ex-vivo biaxial inflation setting. The characterization of the mechanical performance of fetal membrane sealing materials is challenging due to the highly variable physical and biological properties of chorio-amniotic membranes, resulting from anisotropic and inhomogeneous tissue specimen. Therefore, elastomeric membranes were used instead of fresh fetal membrane tissue as model for the experimental evaluation of the

mussel-mimetic tissue adhesive and its repair capabilities. Defects were created and glued with mussel-mimetic sealant under standardized conditions before the sealing performance was evaluated during biaxial inflation testing.

The final study evaluates the sealing performance of mussel glue versus fibrin glue on fresh semi-wet fetal membranes during biaxial inflation testing. The objective was to quantitatively compare the mechanical failure behaviour of human fetal membranes between intact, punctured and sealed (mussel and fibrin glue) membranes on this new membrane inflation device. Additionally, the in vitro plug stability was assessed by incubation of the two glue materials in cell culture media, supplemented with well-defined amounts of enzymes, e.g. collagenase or plasmin.

The envisioned aim is to evaluate mussel glue as a potential candidate material for the fetal membrane sealing after iPPROM in vivo.

2. Results

2.1. Manuscript I

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Biomechanical characterizations of the fetal membranes rupture strength and its relation to microstructural components

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Personal contribution: Design and conduction of all experiments all experiments; biaxial inflation with Ajit S. Mallik (AS) & Julien Egger (JE), biochemistry & histology with AS, mechanics with strong support of JE. Statistics & writing manuscript.

Abstract

Objective

Understanding the fetal membrane (FM) mechanical properties and mechanism leading to their rupture would help to address the repair of preterm prelabour ruptured FM (PPROM). Here, the mechanical properties of amnion, chorion and intact FM are evaluated under physiological conditions and correlated to microstructural parameters.

Study Design

Mechanical parameters of intact FM or separate amnion and chorion membranes were tested in a biaxial biomechanical device and subsequently correlated with collagen and elastin.

Results

The stiffness of amnion is larger than the one of chorion and correlates with its collagen content. Chorion significantly contributes to the critical tension of FM. The critical stretch of intact FM is larger than the sum of the one of amnion and chorion.

Conclusions

Although amnion is the main load bearing membrane, chorion considerably contributes to mechanical properties of the FM. The interaction of amnion and chorion might modulate the ductile behavior of the membranes.

Introduction

Intact fetal membranes (FM) are important to maintain the amniotic fluid homeostasis and to defend against infections. The physiological rupture of the FM occurs as consequence of different factors including mechanical modifications, remodeling of extracellular matrix components, and apoptosis of amnion cells[1]. However, spontaneous preterm prelabour rupture of FM (PPROM) affects 3% of all pregnancies worldwide[2, 3]. This dependent on the gestational age, is associated with a high risk for perinatal morbidity and mortality.

A better understanding of PPROM and the development of membrane sealing strategies requires the knowledge of normal membrane physiology, the investigation of structure function relationship, and the identification of the factors that favor failure of the membranes early in pregnancy.

In order to investigate the biophysical characteristics of the bilayered FM, different methodologies have been applied. For example using a uniaxial tensile test system, membranes have been stretched till rupture[2, 4-8]. In biaxial puncture testing devices, membranes are held in a circular part and load is applied by a spherical indenter. Thereby, the center of the circular probe is moved in direction perpendicular to the membrane till rupture[2, 9-14]. As in the natural situation FM are stretched in all directions of the membrane surface, in the uniaxial tensile experiment the contribution of differently oriented matrix fibers on deformability and force transmission does not correspond to the physiological reponse[6-8, 15]. In the biaxial puncture tests, instead of evenly distributed forces, as in the case of loading from internal liquid pressure, the local force application leads to a state of deformation and mechanisms of membrane rupture that differ significantly from the in-vivo biomechanical loading conditions. Membrane inflation, using air- or fluid pressure to stretch a membrane, best mimics the physiological situation and the in vivo mechanical deformation and physiological rupture events[11, 16, 17]. The aim of the present study was to characterize FM using an inflation experiment, and to correlate the parameters characterizing mechanical failure of intact FM, amnion or chorion membranes with their biochemical composition.

Materials and Methods

Intact FM, as well as blunt dissected amnion and chorion, were tested regarding biomechanical, biochemical and histological parameters. A biomechanical inflation device which allows biaxial loading of tissue samples under close to physiological conditions was employed to simultaneously acquire pressure and geometrical parameters identifying the deformation state of the membrane (Figure 1). These data were used together with histologically determined membrane thickness values to characterize the critical loading conditions of each membrane and correlate the corresponding mechanical parameters with the biochemical composition.

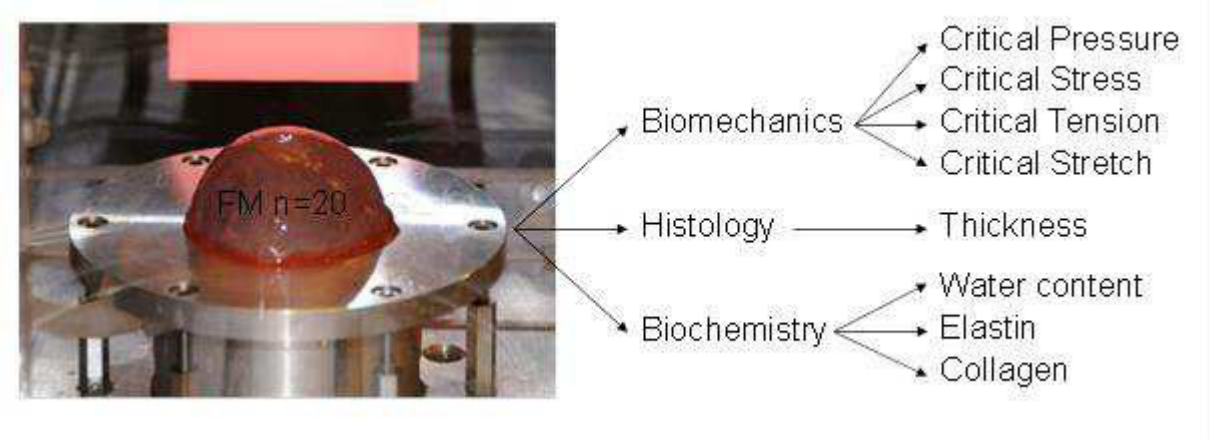


Fig 1. Summary of the main steps of the fetal membrane protocol.

FM samples

FM were collected from patients who underwent elective caesarean section between 37 and 38 weeks of gestation. Patients were recruited for this study with informed written consent using a protocol approved by the Ethical Committee of the District of Zurich (study Stv22/2006). The pregnancies were randomly selected after excluding streptococcus B, hepatitis B or HIV infections. The selected pregnancies had no history of diabetes, connective tissue disorders, and chromosomal abnormalities. After cutting the FM 2 cm away from the placental disc the resulting membrane pieces of 150–200 cm^2 were washed in PBS (phosphate-buffered saline, pH 7.2, without calcium/magnesium).

Design and setup of the inflation device

Our own-built device generates a state of equi-biaxial stress in the central region of the circular samples. Membrane samples are mounted onto the fluid-filled aluminum cylinder

with a 50 mm inner diameter and clamped by a cover ring. The local geometry of the clamping ring is optimized in order to minimize the occurrence of membrane rupture at the samples periphery. The cylinder is connected to an inlet and outlet tubing. The inflow of water is driven by a peristaltic pump (Watson-Marlow Ltd., Zurich, Switzerland) which is computer controlled and allows flow rates of max. 75 mL/min. The increasing fluid pressure inside the cylinder leads to the inflation of the FM samples. The pressure is constantly recorded via a hydrostatic pressure sensor (digital manometer, LEX 1, accuracy 0.05 %, Keller, Switzerland) which is connected to the fluid-filled cylinder. The inflation process is optically monitored by cameras (Point Grey, 1.4 MP Color Grasshopper 1394b Camera, 2/3" CCD) mounted on top and on the side of the cylinder.

Biaxial stretching of FM samples

A total of 20 FM were used either intact or after separating amnion and chorion by blunt dissection to cut out specimen of 70 mm diameter. The resulting membrane samples were glued (UHU glue, 707305, UHU AG, Schönenwerd, Schweiz) in a relaxed state between two sandpaper rings of 50 mm inner and 70 mm outer diameter. The mounted samples were immobilized on the cover ring and clamped onto the water-filled cylinder of the inflation device. The intact FM were placed such that the amnion was in contact with the fluid in the cylinder and the chorion on the outside. The cover ring was fixed using a dynamometric screwdriver (20 Ncm) to reach an equal clamping force at each screw. The loading experiment was performed by applying a constant flow of 0.5 mL/min and by this continuously increasing the pressure in the cylinder until rupture of the membrane sample. The tissue deformation was tracked by the digital images from the side camera. The membrane profile extracted from each image provided information on the state of deformation associated with the corresponding value of internal pressure.

Estimation of the membrane thickness

Membrane thickness measurements were performed as described[15]. In short, tissue samples of intact FM as well as separate membrane layers were embedded in paraffin and 4 µm thick cross-sections were cut using the rotation microtom (HM340E, Microtom GmbH, Walldorf, Germany). Haematoxyline & eosine stained histological sections were analyzed with a Zeiss Axiovert 200M microscope and measurements were directly performed on acquired images

by using Axiovision software (Carl Zeiss, Rel 4.5, Service Package 1). Per membrane mean values of 5 measurements were reported.

Calculation of biomechanical parameters

The loading conditions causing the membrane to fail might be characterized with the value of internal pressure, i.e. the critical pressure (mbar). This parameter describes the level of mechanical loading perpendicular to the membrane surface, but does not characterize the force transmitted through the membrane when rupture occurred. The state of mechanical loading and deformation of the central region of the membrane sample can be assimilated to the one of a sphere subjected to internal pressure. The membrane tension T (force per unit length, N/mm) can be calculated, based on static equilibrium, from the internal pressure p (measured with the pressure sensor) and the radius of membrane curvature r (extracted from the camera image) using the so called Laplace law:

$T=pr/2$

Critical tension values were determined using the values of pressure and curvature at membrane rupture. The critical tension is a structural parameter which characterizes the mechanical resistance of the membrane. In order to determine a material specific mechanical parameter, which might be correlated with specific (gram per total weight) values of collagen or elastin content, the corresponding values of critical stress were calculated. The average stress (force per unit area, N/mm^2 = MPa) was obtained dividing the membrane tension by the current thickness t_0 of the membrane:

$\sigma=T/t_0$

The thickness values t_0 were determined as described in section “Estimation of the membrane thickness”. The current value of membrane area stretch was estimated using the assumption of volume preservation, which is common when analyzing soft biological tissues. Critical stress values were obtained from the values of critical tension and the corresponding thickness at rupture. One additional relevant parameter characterizing the critical mechanical loading conditions is the level of deformation measured immediately before rupture, i.e. the critical stretch. This parameter describes the ratio between the current length of material lines and the corresponding undeformed length. It was determined from the deformed profile and the reference undeformed membrane profile extracted from the images of the lateral camera.

Tissue lyophilisation and water content

Approximately 0.6 g membrane tissue was frozen in liquid nitrogen and was subsequently lyophilized. The water content can be calculated from weighing both wet and dry tissues, whereas the wet weight of the corresponding tissue was set to 100 %.

Determination of the elastin content

Insoluble elastin was extracted from membranes as soluble cross-linked polypeptides by oxidation with oxalic acid. Approximately 50 mg of each lyophilized tissue was extracted three times in 2 mL of 0.25 M oxalic acid by boiling at 100 °C for 1 hour. The three extracts were pooled and the amount of soluble elastin was determined colorimetrically using a Fastin Elastin assay kit (Biocolor Ltd., Newtownabbey, Northern Ireland) following the manufacture's instruction. The color substrate 5,10,15,20-tetraphenyl-21,23-porphyrine after binding to elastin, can be co-precipitated and finally measured at 540 nm absorption. Using standard human alpha elastin, supplied with the kit, the amount of elastin per membrane sample dry weight was determined.

Estimation of the collagen content

For the estimation of the total collagen, an acid hydrolysis method was applied, using 6 N hydrochloric acid (HCl), to determine the hydroxyproline as described earlier [18]. Briefly, 10 mg lyophilized tissue was hydrolyzed with hydrochloric acid (120 °C and for 20 hours), leaving hydroxyprolines intact. 50 µL hydrolyzed collagen was converted with 450 µL Chloramine T (Sigma Aldrich) at room temperature for 25 min to pyrrole-2-carboxylic acid. This was further reacted with 4-dimethylaminobenzaldehyde in propan-2-ol and perchloric acid at 60 °C for 20 min to give as per the amount of hydroxyproline a red discolored product, whose absorbance was measured at 540 nm. An aqueous solution of trans-4-hydroxyproline (trans-4-Hydroxy-L-prolin, Sigma Aldrich, Catno: H5,440-9) was used as standard.

Correlation of critical stress and microstructural components

Correlation coefficients were calculated between microstructural constituents (elastin, collagen in mg/g dry weight) and the critical stress (in MPa). Regression lines between critical stress and elastin or collagen were calculated for intact membranes, amnion and chorion.

Evaluation and statistical analysis

All data are shown as mean ± SD. Box plots ranging from 25th to 75th percentile including the whiskers 10th to the 90th percentile. Statistics was performed using SPSS (PASW statistics 18).

Results

FM samples from 20 elective cesarean sections were evaluated to find correlations between the mechanical parameters and corresponding histomorphometrical (thickness), biochemical (collagen, elastin content) parameters. These samples were either intact fetal membranes or separated individual components as amnion and chorion (Figure 1).

Biaxial stretching of the FM samples

The total surface area of 150 - 200 cm^2 allowed to harvest of 5 ± 1 samples from each FM from a single cesarean section. In about 30 % of all samples rupture of the membranes was observed at the rim of the inflation device. Such samples were excluded from further evaluations as the rupture might be caused by local clamping effects instead of membrane properties. This criterion leads to the evaluation of 12 out of 15 intact FM. In these membranes the mean critical pressure was between 90.3 and 201.6 mbar and resulted in an overall mean of 144.2 ± 42.9 mbar (Table 1). There was no clearly detectable trend regarding a rupture sequence of amnion and chorion, and in some experiments rupture appeared to be simultaneous.

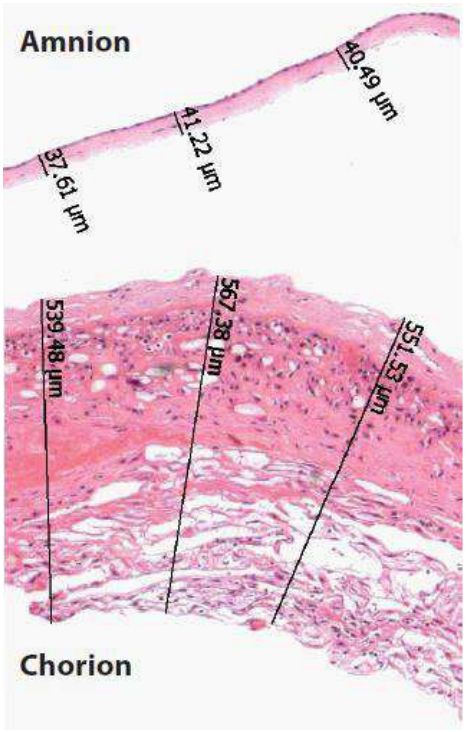
	n=	Pressure [mbar]	Stress [MPa]	Tension [N/mm]	Critical Stretch [-]	Thickness [µm]	H_2O - content [%]	Collagen [mg/g]	Elastin [mg/g]
FM	12	144.2 ± 42.9	1.052 ± 0.881	0.244 ± 0.093	1.223 ± 0.049	513.3 ± 193.4	86.3 ± 1.7	178.8 ± 43.2	176.7 ± 18.5
Amnion	5	111.8 ± 45.1	4.647 ± 2.293	0.199 ± 0.077	1.134 ± 0.029	77.3 ± 14.5	93.8 ± 1.2	296.3 ± 93.0	211.7 ± 24.7
Chorion	5	65.3 ± 21.3	0.639 ± 0.246	0.113 ± 0.037	1.183 ± 0.057	345.6 ± 97.1	91.8 ± 1.5	142.6 ± 46.4	204.7 ± 18.3

Table 1. Measured and calculated mechanical properties after biaxial stretching, histological data and biochemical components for the separated amnion and chorion as well as for the intact fetal membranes. Values are given as MEAN ± SD.

In order to determine the contribution of amnion and chorion to the overall mechanical strength of FM, five blunt dissected individual amnion and chorion membranes were subjected to biaxial inflation. The determined mean critical pressures for amnion and chorion varied between 61.0 and 176.4 mbar and 37.2 and 85.9 mbar respectively.

Overall the achieved mean pressure in our evaluation is twofold higher for amnion membranes 111.8 ± 45.1 mbar than for chorion membranes 65.3 ± 21.3 mbar (Table 1). These results indicate a significant contribution of chorion to the membrane mechanics.

Determination of the membrane thickness



To determine the materials properties of the individual membranes, a precise membrane thickness measurement is mandatory. Thus, haematoxyline & eosine stained tissue sections were used to determine the thickness of the individual membrane layers or the intact FM (Fig. 2). For the intact FM, an average thickness of 513.3 ± 193.4 µm was shown. Amnion was demonstrated to be 77.3 ± 14.5 µm and chorion 345.6 ± 97.1 µm in thickness (Table 1).

Fig. 2 Haematoxyline and eosine staining of the amnion (top layer) and the chorion (bottom layer). Thickness measurements were performed as indicated by the numbers.

Calculation of biomechanical parameters

The critical stress, a parameter representative of materials strength properties, achieved 1.052 ± 0.881 MPa for the intact membrane samples. Compared to the 4.647 ± 2.293 MPa reached by separately measured amnion membranes, this is a relatively low value, which is due to the lower critical stress values of the chorion membranes of 0.639 ± 0.246 MPa (Fig. 3). Thus, the FM is a layered structure of large materials strength on the amnion side and lower materials strength on the chorion side.

The mechanical resistance of the membrane, which is the relevant parameter in terms of physiological loading conditions, is characterized through the critical tension. For this parameter, intact FM samples achieved 0.244 ± 0.093 N/mm. The values of the individual layers with 0.199 ± 0.077 N/mm for the amnion and 0.113 ± 0.037 N/mm for the chorion showed that the chorion membrane significantly contributed to resist membrane tension (Figure 4). This result is confirmed in terms of critical pressure, with the intact membrane resisting significantly higher values of internal pressure as compared to the amnion alone. The

mean critical pressure of the intact membranes is comparable to the sum of the averages measured with individual amnion or chorion samples (Table 1).

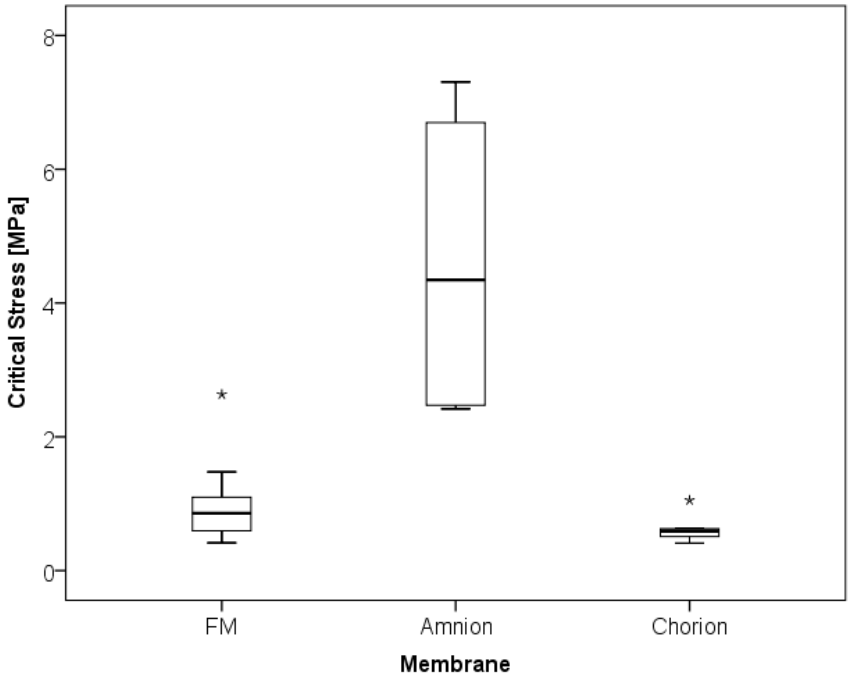


Fig 3. Comparison of the critical stress between the intact fetal membranes (FM, n=12) and the separate amnion (n=5) and chorion (n=5) after biaxial inflation. Amnion shows to withstand a higher stress level compared to chorion and the intact fetal membranes.

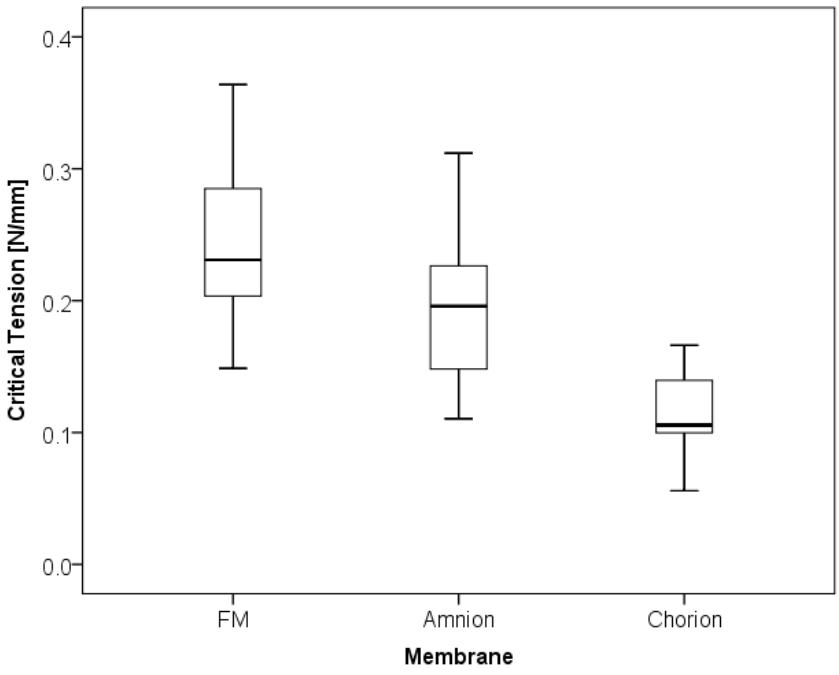


Fig 4. Comparison of the critical tension between the intact fetal membranes (FM, n=12) and the separate amnion (n=5) and chorion (n=5) after biaxial inflation. The intact fetal membrane shows to resist the highest tension.

As not only the mechanical strength but also the deformability of the membrane material is important for the in-vivo rupture of FM, the critical stretch was evaluated. Interestingly, the intact FM showed highest values 1.223 ± 0.049 followed by chorion 1.183 ± 0.057 and amnion 1.134 ± 0.029 (Figure 5). These results indicate that chorion can undergo larger deformations than amnion, and that the interaction of the two layers in the intact membrane allowed higher deformations to occur as compared to the amnion alone.

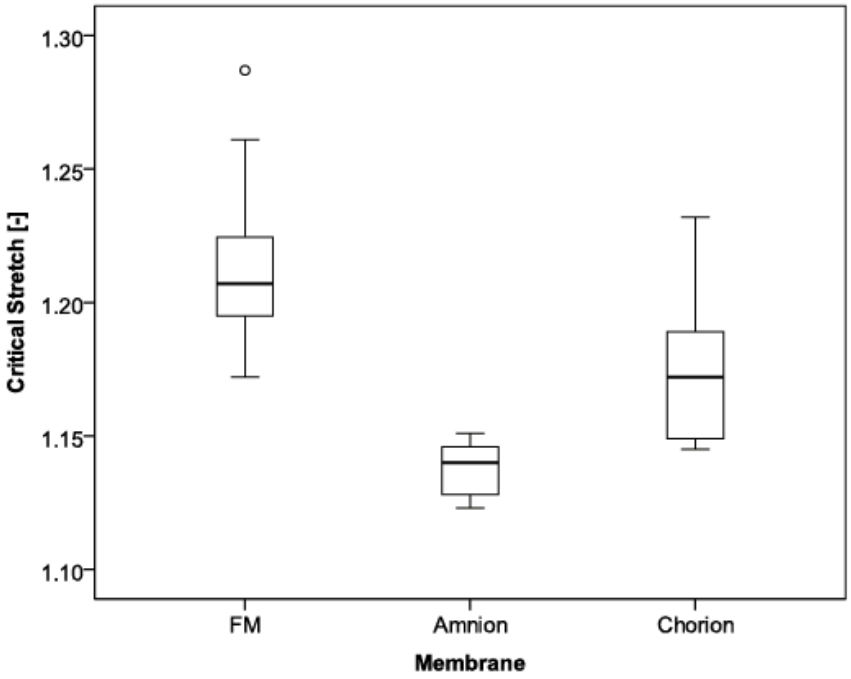


Fig. 5 Comparison of the critical stretch between the intact FM (FM, n=12) and the separate amnion (n=5) and chorion (n=5) after biaxial inflation. The intact FM results in the highest extension. However, both of the separate layers contribute to the stretch, as shown by theirs separate values. Chorion presents the more elastic layer.

Evaluation of the elastin and the collagen content of FM

In order to evaluate the correlation between membrane composition and membrane mechanical properties of the two main components of the extracellular matrix, total collagen and total elastin were determined. The overall elastin content was not significantly different in the chorion (204.7 ± 18.3 mg/g), amnion (211.7 ± 24.7 mg/g) and consequently in the intact membrane (176.7 ± 18.5 mg/g) (Table 1).

In contrast, the collagen content of the amnion was twofold higher (296.3 ± 93 mg/g) than the one of the chorion (142.6 ± 46.4 mg/g). This inhomogeneous distribution of the collagen resulted in an overall content of 178.8 ± 43.2 mg/g for the intact membrane.

Correlation of the biochemical components to the mechanical strength

Next, the elastin and collagen were related to the critical stress values. Relatively good correlations of collagen content and critical stress were found for the intact FM ($r^2 = 0.6866$) (Figure 6A) and the amnion ($r^2 = 0.7446$) (Figure 6B), whereas in chorion the mechanical measurements do not correlate with the collagen content (Figure 6C). This indicates that collagen structures contribute critically to the strength of the amnion membranes whereas they are not determining the mechanical stability of the chorion.

Fig. 6A

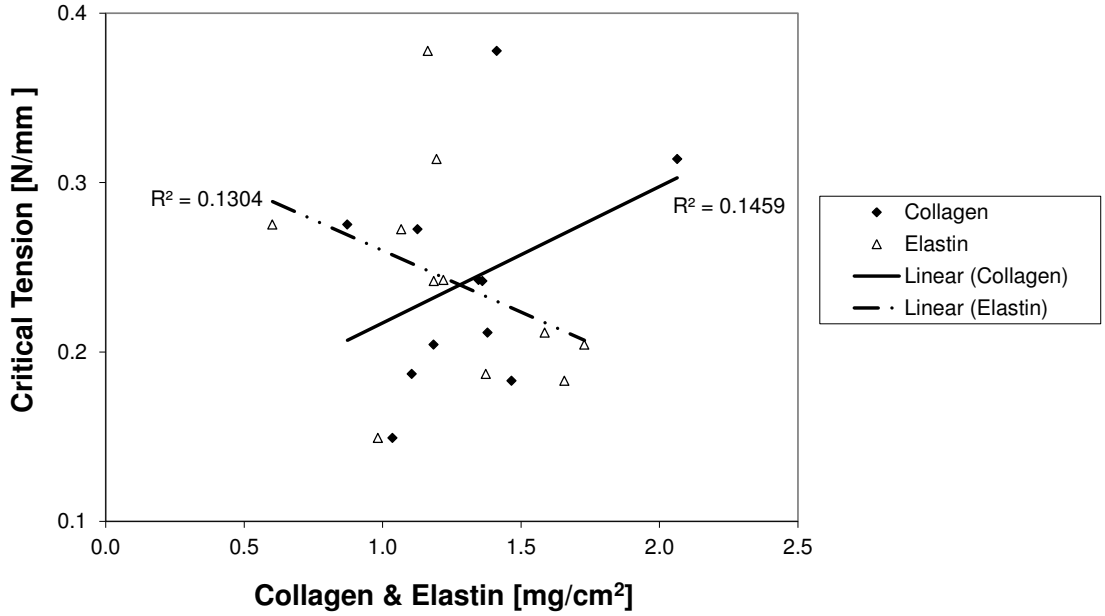


Fig. 6B

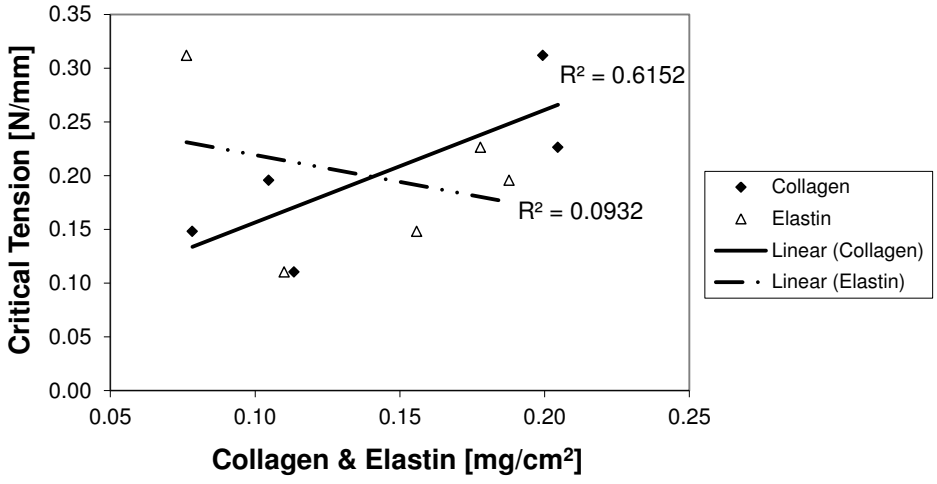


Fig. 6C

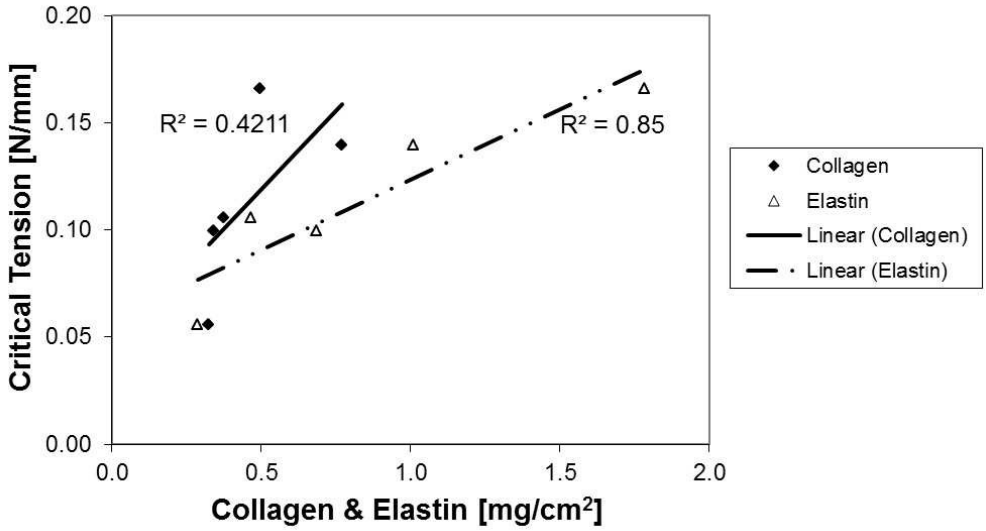


Fig. 6 Correlations of the critical tension to collagen & elastin presented in mg/g. A) For the intact FM correlation was observed neither for elastin nor for collagen. B) In amnion, the correlation of collagen to the critical tension is r^2 = 0.6152. C) In chorion, the relation of elastin to the critical tension shows a correlation coefficient of r^2 = 0.85.

No correlation of elastin to the critical stress values could be found in the intact FM (r^2 = 0.0154), the amnion (r^2 = 0.0052), or the chorion (r^2 = 0.1346).

Discussion

This study demonstrates that under physiological loading conditions the collagen content contributes to the strength of the amnion membrane. It furthermore shows that chorion in conjunction with amnion plays an important, often underestimated role for FM stability.

Earlier studies characterizing FM by uniaxial methods[5-8, 19] or biaxial puncture testing[2, 10, 12, 13, 20] provided important fundamental information. Here, by using a biaxial inflation approach, a close simulation of the in vivo situation of FM is achieved in terms of loading and rupture conditions. By individual measurement of amnion and chorion, amnion is confirmed to be the load-bearing layer by withstanding higher tension values despite a four times lower thickness as compared to the chorion[7, 9, 13, 20]. However, regarding the resistance to physiological loading conditions, the present data indicates that both layers, the thin and strong amnion and the thicker and more compliant chorion, contribute to membrane's strength.

Critical pressure values indicate the importance of the interconnection between the two layers[9, 13, 21]. During inflation experiments, the more compliant chorion layer might locally support initial lesions of amnion, causing final rupture to occur at higher level of pressure, membrane tension and deformation as compared to the amnion layer alone.

The rupture sequence of the membranes was observed by eye and all events were qualitatively described (data not shown). In contrast to Arikat et al, who described that the choriodecidua fails consistently before amnion does, we found membranes where larger lesions of amnion occurred before chorion ruptured and others where the opposite was observed or both membranes ruptured at the same location simultaneously. This discrepancy with respect to the work of Arikat et al might be explained by the different rupture criteria and the applied biaxial puncture technique. In this technique local interactions between membrane and plunger, and a deformation state in the vicinity of the plunger which might influence the rupture sequence; furthermore, rupture in their work was related to a diminished puncture force, whereas in our case rupture was identified by the occurrence of water leakage through local tissue lesions.

A limitation in previous studies about physical parameters of FM has been the lack of the membrane thickness measurement, an important parameter for determining material specific mechanical properties[2]. In the present work the measured thicknesses of separated layers[15] were considerably larger than reported previously in the literature[6, 7, 22], whereas the relative values of amnion with respect to chorion correspond with previous findings. The two layers

are only passively attached to each other and a potential space between amnion and chorion has been reported[23]. Due to these facts, we speculate that an appropriate separation of amnion and chorion can be achieved by peeling. Nevertheless, an uncertainty about correct separation as well as about the thickness measurement remains and has to be evaluated in more detail in future work.

The collagen content in the present work was shown to be consistent with the existing literature. On the other hand, the elastin content was found between 18 and 21 % of dry weight throughout all layers of the fetal membranes, which in comparison with the literature is significantly higher. Furthermore, adding the evaluated elastin content of separate amnion and chorion together, a large value for elastin would result compared to the amount of elastin investigated for the intact fetal membranes. Hieber et al. reported elastin values of 0.08 % per fat free dry weight, Jabareen et al. referred to an average value of approximately 10 % elastin per dry weight and Wilshaw et al. presented values of wet weight amnion with 36 % elastin[15, 24, 25]. The provided data is inconsistent and does indicate the great difficulty to quantify elastin in this tissue using biochemical assays. Uncertainties do involve the extraction of the proteins but also the corresponding specificity of the assays.

A positive correlation of collagen and critical stress was determined in amnion and intact FM samples. To our knowledge this is the only work being done about relation of mechanical strength and biochemical parameters in FM in the biaxial model. In the case for chorion, no correlation was observed in the relationship of collagen.

Amnion is the stiffer and stronger layer due to the resistance to large deformation of collagen fibers. Elastin is the elastic component of the FM that contributes to higher deformability such as in the chorionic layer. For elastin no pronounced proportionality was observed to the critical stress in all tested membrane layers. Such a relation was not expected since critical stress is measured at high level of deformation, whereas elastin is associated with the low strain mechanical response[15]. Speculation would hypothesize that a membrane with higher amount of elastin and less amount of collagen behaves as an “elastic” layer, which allows large deformation with low resistance to high pressure. In this sense the chorion complements the amnion, containing mainly collagen and a low amount of elastin, thus representing a stiffer membrane, withstanding high pressures but showing lower extensibility.

In conclusion, the herein presented data demonstrate the importance of biomechanical and biochemical data on FM in order to achieve new knowledge about the events leading to PPROM.

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References

1. OSMAN I, YOUNG A, JORDAN F, GREER IA, NORMAN JE. Leukocyte density and proinflammatory mediator expression in regional human fetal membranes and decidua before and during labor at term. J Soc Gynecol Investig 2006;13:97-103.
2. CALVIN SE, OYEN ML. Microstructure and mechanics of the chorioamnion membrane with an emphasis on fracture properties. Ann N Y Acad Sci 2007;1101:166-85.
3. MERCER BM. Preterm premature rupture of the membranes. Obstet Gynecol 2003;101:178-93.
4. ARTAL R, BURGESON RE, HOBEL CJ, HOLLISTER D. An in vitro model for the study of enzymatically mediated biomechanical changes in the chorioamniotic membranes. Am J Obstet Gynecol 1979;133:656-9.
5. ARTAL R, SOKOL RJ, NEUMAN M, BURSTEIN AH, STOJKOV J. The mechanical properties of prematurely and non--prematurely ruptured membranes. Methods and preliminary results. Am J Obstet Gynecol 1976;125:655-9.
6. HELMIG R, OXLUND H, PETERSEN LK, ULDBJERG N. Different biomechanical properties of human fetal membranes obtained before and after delivery. Eur J Obstet Gynecol Reprod Biol 1993;48:183-9.
7. OXLUND H, HELMIG R, HALABURT JT, ULDBJERG N. Biomechanical analysis of human chorioamniotic membranes. Eur J Obstet Gynecol Reprod Biol 1990;34:247-55.
8. OYEN ML, CALVIN SE, COOK RF. Uniaxial stress-relaxation and stress-strain responses of human amnion. J Mater Sci Mater Med 2004;15:619-24.
9. ARIKAT S, NOVINCE RW, MERCER BM, et al. Separation of amnion from choriodecidua is an integral event to the rupture of normal term fetal membranes and constitutes a significant component of the work required. Am J Obstet Gynecol 2006;194:211-7.

10. EL KHWAD M, STETZER B, MOORE RM, et al. Term human fetal membranes have a weak zone overlying the lower uterine pole and cervix before onset of labor. Biol Reprod 2005;72:720-6.
11. LAVERY JP, MILLER CE, KNIGHT RD. The effect of labor on the rheologic response of chorioamniotic membranes. Obstet Gynecol 1982;60:87-92.
12. MOORE RM, MANSOUR JM, REDLINE RW, MERCER BM, MOORE JJ. The physiology of fetal membrane rupture: insight gained from the determination of physical properties. Placenta 2006;27:1037-51.
13. OYEN ML, CALVIN SE, LANDERS DV. Premature rupture of the fetal membranes: is the amnion the major determinant? Am J Obstet Gynecol 2006;195:510-5.
14. PRESSMAN EK, CAVANAUGH JL, WOODS JR. Physical properties of the chorioamnion throughout gestation. Am J Obstet Gynecol 2002;187:672-5.
15. JABAREEN M, MALLIK AS, BILIC G, ZISCH AH, MAZZA E. Relation between mechanical properties and microstructure of human fetal membranes: an attempt towards a quantitative analysis. Eur J Obstet Gynecol Reprod Biol 2009;144 Suppl 1:S134-41.
16. LAVERY JP, MILLER CE. The viscoelastic nature of chorioamniotic membranes. Obstet Gynecol 1977;50:467-72.
17. MACLACHLAN TB. A Method for the Investigation of the Strength of the Fetal Membranes. Am J Obstet Gynecol 1965;91:309-13.
18. SKINNER SJ, CAMPOS GA, LIGGINS GC. Collagen content of human amniotic membranes: effect of gestation length and premature rupture. Obstet Gynecol 1981;57:487-9.
19. OYEN ML CR, STYLIANOPOULOS T, BAROCAS VH, CALVIN SE, LANDERS DV. Uniaxial and biaxial mechanical behaviour of human amnion. . J Mater Res 2005;20:2902-9.
20. OYEN ML, COOK RF, CALVIN SE. Mechanical failure of human fetal membrane tissues. J Mater Sci Mater Med 2004;15:651-8.
21. KUMAR D, NOVINCE R, STROHL A, et al. A new methodology to measure strength of adherence of the fetal membrane components, amnion and the choriodecidua. Placenta 2009;30:560-3.

22. PRÉVOST TP. Biomechanics of the Human Chorioamnion, Department of Materials Science and Engineering: Massachusetts Institute of Technology, 2006 (vol Master of Science).
23. ERNST LM. Color atlas of fetal and neonatal histology. New york: Springer Science+Business Media, LLC; Number of pages.
24. HIEBER AD, CORCINO D, MOTOSUE J, et al. Detection of elastin in the human fetal membranes: proposed molecular basis for elasticity. Placenta 1997;18:301-12.
25. WILSHAW SP, KEARNEY JN, FISHER J, INGHAM E. Production of an acellular amniotic membrane matrix for use in tissue engineering. Tissue Eng 2006;12:2117-29.

2.2. Manuscript II

Mussel-mimetic tissue adhesive for fetal membrane repair: a standardized ex vivo evaluation using elastomeric membranes

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Personal contribution:
Design and conduction of all experiments all experiments with substantial support in mechanical evaluations of Wilfried Bürzle (WB). Statistics & writing manuscript.

Link to supporting material:
http://onlinelibrary.wiley.com/store/10.1002/pd.2712/asset/supinfo/pd2712_suppinfomovie.mov?v=1&s=dfcc2ea6a7b15c0f8e7ad97ea35c10fe18e693a8

Abstract

Objective

Iatrogenic preterm premature rupture of membranes (iPPROM), the main complication of invasive interventions in the prenatal period, seriously limits the benefit of diagnostic or surgical prenatal procedures. This study aimed to evaluate preventive plugging of punctured fetal membranes in an ex vivo situation using a new mussel-mimetic tissue adhesive (mussel glue) to inhibit leakage.

Methods

A novel biomechanical test device that tests the closure of injured membranes under near-physiological conditions was used. Mussel glue, a poly(ethylene glycol)-based hydrogel, was used to seal membrane defects of up to 3mm in mechanically well-defined elastomeric membranes with three different degrees of stiffness.

Results

Elastomeric test membranes were successfully employed for testing mussel glue under well-defined conditions. Mussel glue plugs were distended by up to 94%, which translated to an improved sealing efficiency on elastomeric membranes with high stiffness. For the stiffest membrane tested, a critical burst pressure of 48mbar (36mmHg) was accomplished in this ex vivo setting.

Conclusions

Mussel glue appears to efficiently seal membrane defects under well standardized ex vivo conditions. As repaired membranes resist pressures measured in amniotic cavities, mussel glue might represent a novel sealing method for iatrogenic membrane defects.

Introduction

With increasing numbers of invasive interventions in the prenatal period for diagnostic or therapeutic purposes, the iatrogenic preterm pre-labor rupture of fetal membranes (iPPROM) becomes more and more important. The healing potential of fetal membranes is very limited, or even absent, as shown by a histological follow-up study. In fact, several months after the intervention, fetoscopic puncture sites had similar extension and showed no obvious signs of healing. Rather, spontaneous closure of needle punctures may be attributed to the relative sliding of the amnion and chorion against one another. This could also be due to the remaining attachment of the chorion to the decidual layer at the uterine wall by means of fibrin or any other crust associated with a healing process (Gratacos et al., 2006).

In monochorionic twin pregnancies, prenatal interventions like the selective laser coagulation of placental anastomoses are widely-accepted treatments for twin-to-twin transfusion syndrome (TTTS) (Senat et al., 2004). Further developments in prenatal invasive procedures provide promising therapeutic options for other fetal diseases, such as congenital diaphragmatic hernia (CDH), by placing a balloon into the fetal trachea at 26 weeks of gestation with fetoscopic removal at 34 weeks (Jani et al., 2009). However, such potentially beneficial interventions might be seriously limited by the relatively high occurrence of iPPROM after fetoscopic intervention, ranging between 4% and 100% (Deprest et al., 2010). Severe complications resulting from premature birth include respiratory distress syndrome (RDS), cerebral palsy, blindness, deafness, kernicterus, or necrotizing enterocolitis.

Today, treatment options to restore fetal membrane integrity are very limited; further, none have made it into clinical practice. Several strategies to repair fetal membranes, such as plugging, biological healing, or sealing by surgical glues, were evaluated (Devlieger et al., 2006). Attempts to seal the fetoscopic entry site by collagen scaffolds might be limited by the stability of the matrix, resulting in a lack of long-term stability. Based on the hypothesis that

cell instructive scaffolds can induce a biological repair, decellularized amnion tissue was evaluated in a rabbit gestational model (Mallik et al., 2007, Ochsenbein-Kolble et al., 2007). Although some endogenous cells were recruited to the matrix plug during the postoperative phase of the experiment, the sealing of the fetal membrane mainly relied on closure of the puncture site by the plug (Mallik et al., 2007, Ochsenbein-Kolble et al., 2007). The limited healing capacity of fetal membranes and the instability of plugging materials might limit the practicality of these approaches. Tissue adhesives that exhibit efficient, non-disruptive, and non-toxic bonding to fetal membranes under wet gluing conditions might be a more viable alternative.

The group of Phillip Messersmith has described the formation of a hydrogel glue by conferring an ability of marine blue mussels to firmly adhere to a wide variety of materials upon poly(ethylene glycol) (PEG) (Lee et al., 2002, Lee et al., 2007). This mussel mimetic tissue adhesive (mussel glue) is based on branched PEG, which is functionalized with the rare amino acid residue 3,4 dihydroxyphenylalanine (DOPA) present in high concentrations in mussel foot proteins (Lee et al., 2002, Lee et al., 2007, Waite, 1999, Waite and Tanzer, 1980). By conversion of DOPA under oxidative conditions, highly reactive DOPA-quinone is formed, allowing native mussel adhesive protein adhesion to take place in a wet, normal saline environment. A PEG polymer containing a DOPA analogue in the form of a reactive catechol group (cPEG) (Brubaker et al., 2010, Burke et al., 2007, Lee et al., 2002, Messersmith, 2008, Yamada et al., 2000) has not shown any signs of cytotoxicity in vitro (Bilic et al., 2010) and has also been successfully employed in in vivo studies (Brubaker et al., 2010). Therefore, mussel glue seems to be a potential candidate for sealing fetal membrane defects and preventing amniotic fluid leakage.

Thus, the aim of the present study was to develop a standardized regimen to test the properties of mussel glue for the sealing of fetal membranes. We developed an experimental setup that

allows for the loading of membranes in a multi-axial stress state through the application of a fluid pressure on one side of a circular membrane test piece. This setup generates loading conditions that are more physiologically representative compared to the well known ball-burst tester, a setup that induced a localized equi-biaxial stress field through a spherical indenter punching the membrane (El Khwad et al., 2005). As the physical and biological properties of chorioamniotic membranes are highly variable, characterization of the mechanical performance of sealing materials might be largely hindered by non-homogenous samples. Therefore, elastomeric membranes were used as model for the experimental evaluation of the mussel-mimetic adhesive and its repair capabilities. The elastomeric membranes consist of the acrylic elastomer VHB 4910, which is characterized by high extensibility and a pronounced time dependence of its mechanical response. VHB 4910 has been recently employed for the realization of electro-active polymer actuators, also called "artificial muscles" (Bar-Cohen, 2004). Here, in a series of elastomeric membranes with three different mechanical degrees of stiffness, defects were created and glued with mussel glue under standardized conditions.

Materials and Methods

Mussel mimetic sealant

The production and characterization of mussel glue, a catechol-functionalized poly(ethylene glycol) (cPEG) lacking the primary amine of the DOPA amino acid, was performed as described elsewhere (Brubaker et al., 2010). For the formation of hydrogels, equal volumes of cPEG precursor solution (300 mg/mL in phosphate-buffered saline (PBS) and 0.01% wt Brilliant Blue (Shanghai Chemorole Ltd)) and sodium periodate solution (12 mg/mL in water) were mixed by pipetting. Sodium periodate initiated gelation, which was achieved when reactive dopamine groups formed covalent cross-links (Lee et al., 2002). This was allowed to proceed for five minutes at room temperature.

Design and setup of the loading device

A custom-built device to mechanically stretch membranes in a biaxial way was used. The device consisted of an aluminum cylinder with a 50mm inner diameter that was mounted by clamping membrane samples between the cylinder and a cover ring (Figure 1). The cylinder was connected to both inlet and outlet tubing. The membranous tissue was inflated on the water-filled cylinder using a peristaltic pump (type 314VBM, four rollers, max. 360rpm, Watson-Marlow Ltd., Zurich, Switzerland), which was computer-controlled and allowed flow rates of 75mL/min. maximum. The pressure generated by increasing the liquid volume, using constant flow, was measured with a hydrostatic pressure sensor (digital manometer, LEX 1, -1 to 2bar, accuracy within 0.05%, Keller, Switzerland) positioned at the outlet of the cylinder. The deformation of the membrane was continuously monitored by video cameras (Point Grey, 1.4MP Color Grasshopper 1394b Camera, 2/3" CCD) mounted on the top and side of the cylinder.

Stiffness comparison of fetal membranes and elastomeric membranes

A total of 10 fetal membranes were collected with written consent at elective caesarean sections. Mean gestational age was 38 ± 1 weeks in the absence of labor, preterm rupture of membranes, chorioamnionitis, or chromosomal abnormalities. Fetal membranes and very high bonding elastomeric membranes (3M AG, Rüschlikon, Switzerland) of 0.5 and 1mm in thickness were cut to a diameter of approximately 7cm. To obtain elastomeric membranes of 2mm thickness, two 1mm elastomeric membranes were bonded together by simply utilizing their inherent bonding capacity. The resulting 0.5, 1.0 and 2.0mm thick elastomeric and the fetal membranes were immobilized on the cover ring and clamped onto the water-filled cylinder of our loading device. The loading experiment was performed by applying a constant

flow rate of 12mL/min until the pressure reached 10, 20 or 40mbar. The height of the apex was evaluated by measurements from images taken with the side camera.

Elastomeric membrane gluing and mechanical testing

Very high bonding elastomeric membranes of 0.5, 1.0 and 2.0mm thickness were immobilized using the cover ring of the loading device. All elastomeric membranes were punched using a punch device (BP-30F, kai medical). 150µL of mussel glue was applied in a lubricated mold, resulting in a cylindrical plug with a thickness of 1.4mm and diameter of 12.9mm. The traumatized and glued elastomeric membranes were then clamped onto the water-filled cylinder by tightening the screws using a dynamometric screwdriver at 0.2Nm. Finally, the loading experiment was performed by applying a constant flow rate of 12mL/min until the elastomeric membranes ruptured. The membranes were continuously monitored optically and pressures were recorded.

Evaluation and statistical analysis

The critical burst pressure corresponds to the highest achieved pressure before rupture of the elastomeric membrane. Before the onset of inflation, diameters of the mussel glue plug (P_0) and the defect size (D_0) were measured and compared to the diameters of the plug (P_1) and the defect (D_1) after stretching (referring to the critical state just prior to rupture). Data are shown throughout as [mean ± SD]. Statistics were performed by the Kruskal Wallis und Mann Whitney test using the SPSS program (PASW statistics 18).

Results

Comparison of elastomeric membranes with fetal membranes

To estimate the value of elastomeric membranes for the measurement of glue properties, the viscoelastic behaviors of 0.5, 1, and 2mm elastomeric membranes were compared with fetal membranes (Figure 2). The deformation of the membranes during inflation (between 10mbar and 40mbar of pressure) was determined by measuring the elevation of the apex. The distension of the fetal membranes at 10mbar (3.3 ± 0.3mm height) was very similar to that one of the 2mm elastomeric membranes (2.9 ± 0.3mm). However, at pressures higher than 10mbar, fetal membrane distension was only half as much as that one of the 2mm elastomeric membranes.

Standardization of puncture and sealing procedure

In order to accurately determine the properties of the glue and the influence of the mechanical properties of the elastomeric membrane on plug stability, the formation of the membrane defects and the glue application were standardized (Figure 3A). Membranes with thicknesses of 0.5, 1 and 2mm were punctured with a punch device to generate defined holes with diameters of 2.9 ± 0.2mm at the center of the membrane, which were efficiently and immediately glued to prevent defect propagation upon inflation. To control the extension and sealing geometry, lubricated molds were immobilized and centered on top of the membrane defect prior to the formation of a plug using 125µL of mussel glue. This approach led to the formation of a well-defined cylindrical plug with a diameter of 12.9 ± 0.1mm and thickness of 1.36 ± 0.05mm.

Critical Pressure of traumatized and sealed elastomeric membranes

Next, elastomeric membranes of different thickness (0.5, 1.0 and 2.0 mm) and different defined degrees of stiffness were tested using our biaxial inflation device after standardized defect formation and mussel glue application. A video presentation shows the inflation of a repaired 1mm thick elastomeric membrane until rupture (Supplemental Movie 1). Typical examples of inflated elastomeric membranes just before rupture are also depicted (Figure 3A). The elastomeric membrane thickness, which translated to membrane stiffness, correlated with the size of the formed membrane bubble before rupture. Surprisingly, the critical pressure of mussel glue-sealed elastomeric membranes correlated with the stiffness of these membranes. By increasing the thickness of the elastomeric membranes from 0.5 to 1 and 2mm, the resistance to burst for all groups significantly improved from 12.6 ± 1.6mbar to 22.4 ± 1.7mbar and 45.1 ± 5.5mbar respectively (Figure 3B). (Between the 0.5 and 2.0 mm group, $P \leq 0.01$; between all other groups, $P \leq 0.05$).

Distension of the mussel glue

Based on the repair experiments above, we hypothesized that the performance of mussel glue is dependent on elastomeric membrane deformation. To determine the distension of the mussel glue in response to loading, the increase in diameter of the glue plug (P) and the defect (D) were measured before inflation and immediately before the critical burst pressure was reached (Figure 4A). The diameters of the glue plug and the defect increased 49 ± 34% and 94 ± 55%, respectively, on the 2mm thick elastomeric membranes (Figure 4B). The values were not significantly different between all the evaluated conditions.

Discussion

This study presents data on the successful sealing of 3mm elastomeric membrane defects using the mussel glue, a new promising tissue sealant that polymerizes in seconds and bonds even under wet conditions

In the last few years, several possible strategies to prevent or treat PROM have been described, including collagen plugs (Gratacos et al., 2000), native amniotic scaffolds (Mallik et al., 2007, Ochsenbein-Kolble et al., 2007), collagen plugs enriched with platelets and amniotic fluid cells (Liekens et al., 2008) in a rabbit model or gelatin sponge plugs in a ovine and primate model (Luks et al., 1999), fibrin glue (Sciscione et al., 2001, Harmanli et al., 1998), amniopatches (Quintero, 2001, Quintero et al., 1999, Quintero et al., 1996), amniografts (Quintero et al., 2002), maternal blood clot plugs (Sener et al., 1997), combinations of platelets, fibrin glue and powdered collagen slurry (Young et al., 2004), and gelatine sponges (Papanna et al., 2010) in humans. The relatively poor outcome using collagen-based matrices in conjunction with fibrin glue might be attributed to their high susceptibility to proteolytic remodelling and might be a result of instable plugging and leakage shortly after application (Quintero et al., 2002).

While plugging and healing of fetal membranes are attractive strategies for long-term sealing, we hypothesize that gluing of the membranes with biologically inert materials might be fully sufficient for temporary closure of the trauma site. In an in vivo experiment with diabetic mice, mussel glue was used to stick and engraft pancreatic islets on to the surface of the liver, leading to functional recovery for up to one year (Brubaker et al., 2010) Thus, the temporary presence of sodium periodate, which is readily reduced during chemical crosslinking (Burke et al., 2007), had no adverse effects in vitro and in vivo.

In order to measure the mechanical stability of mussel glue-repaired membranes, we used standardized conditions for membrane defect formation and mussel glue application. The use of elastomeric membranes with reproducible materials properties permitted the evaluation of the glue properties independent of the mechanically highly variable fetal tissue. Although these conditions are considerably different from possible in vivo treatment applications, defined plug geometries in combination with elastomeric membranes of defined viscoelastic properties provide valuable insight to gluing material properties.

Our data show that the critical burst pressure after the repair of 3mm diameter puncture defects is strongly influenced by the mechanical properties of the membrane. Since our 2mm thick elastomeric model membranes at a pressure of 40mbar almost reach the stiffness of fetal membranes, we think that the achieved mechanical stability of the repaired elastomeric membranes might be a good estimation of the expected performance of mussel glue on fetal membranes. Although, we have not performed measurements with more rigid substrates, the results indicate that stiffer fetal membranes might achieve even higher critical burst pressures than the 2mm model membranes after repair with mussel glue.

The critical pressure measurements, along with the almost-identical dilatation seen in all instances immediately before burst, demonstrate that mussel glue in the current formulation and application geometry can be stretched to 94 ± 54% in the defect and 49 ± 34% in the plug area of the 2 mm thick elastomeric membrane before it ruptures. These findings indicate that the deformation behavior of the glued membrane is determined by the mechanical properties of the membrane, while rupture occurs when a critical state of deformation is reached in the glue plug. As a consequence, burst pressure level depends on the stiffness of the membrane and could be significantly higher than the 45.1 ± 5.5 mbar observed with the 2mm thick elastomeric membrane. On the other hand, the present experiments indicate that the highly deformable mussel glue effectively seals the perforated elastomeric membrane, but does not

offer a significant local reinforcement. This means that the glue plug will not protect the membrane from a possible increase in the size of the defect, but is sufficiently stable to resist the local loading generated by the pressure field. Recently, pre-sealing of the fetal membranes prior to fetoscopic surgery was tested in a model using the membrane of unfertilized chicken eggs (Carnaghan and Harrison, 2009). Although, some sealants in these experiments were effective in mitigating fluid leakage and enhancing resistance to shear forces and pressure, clinically-important properties of the materials have yet to be investigated.

The influence of the sealing shape, mussel glue thickness, and tension distribution are all subject to ongoing studies and are expected to leave potential for further optimization of gluing performance. This will be important regarding clinical applications, where the material will initially be engineered to seal right after invasive interventions such as amniocentesis or fetoscopy. Furthermore, it should be noted that the fetal membranes in vivo are reinforced by the surrounding myometrium such that stretch rather than pressure, might be the determining factor.

In conclusion, the data presented herein demonstrate that mussel glue, a new promising tissue sealant that bonds even under wet conditions, can be used to efficiently seal elastomeric membranes with viscoelastic properties comparable to those of fetal membranes. Further ex vivo and in in vivo evaluations are necessary and are already ongoing to confirm the applicability of mussel glue to fetal membrane repair.

Supplemental Information

Supplemental materials to this article can be found online.

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Financial disclosure/competing interest

Dr. Messersmith holds equity in Nerites Corporation, a company that develops surgical sealants and adhesives.

References

BAR-COHEN, Y. 2004. Bionic Humans Using EAP as Artificial Muscles: Reality and Challenges. International Journal of Advanced Robotic Systems, 1: 217-222.

BILIC, G., BRUBAKER, C., MESSERSMITH, P. B., MALLIK, A. S., QUINN, T. M., HALLER, C., DONE, E., GUCCIARDO, L., ZEISBERGER, S. M., ZIMMERMANN, R., DEPREST, J. & ZISCH, A. H. 2010. Injectable candidate sealants for fetal membrane repair: bonding and toxicity in vitro. Am J Obstet Gynecol, 202**,** 85 e1-9.

BRUBAKER, C. E., KISSLER, H., WANG, L. J., KAUFMAN, D. B. & MESSERSMITH, P. B. 2010. Biological performance of mussel-inspired adhesive in extrahepatic islet transplantation. Biomaterials, 31**,** 420-7.

BURKE, S. A., RITTER-JONES, M., LEE, B. P. & MESSERSMITH, P. B. 2007. Thermal gelation and tissue adhesion of biomimetic hydrogels. Biomed Mater, 2**,** 203-10.

CARNAGHAN, H. K. & HARRISON, M. R. 2009. Presealing of the chorioamniotic membranes prior to fetoscopic surgery: preliminary study with unfertilized chicken egg models. Eur J Obstet Gynecol Reprod Biol, 144 Suppl 1**,** S142-5.

DEPREST, J. A., FLAKE, A. W., GRATACOS, E., VILLE, Y., HECHER, K., NICOLAIDES, K., JOHNSON, M. P., LUKS, F. I., ADZICK, N. S. & HARRISON, M. R. 2010. The making of fetal surgery. Prenat Diagn, 30**,** 653-67.

DEVLIEGER, R., MILLAR, L. K., BRYANT-GREENWOOD, G., LEWI, L. & DEPREST, J. A. 2006. Fetal membrane healing after spontaneous and iatrogenic membrane rupture: a review of current evidence. Am J Obstet Gynecol, 195**,** 1512-20.

EL KHWAD, M., STETZER, B., MOORE, R. M., KUMAR, D., MERCER, B., ARIKAT, S., REDLINE, R. W., MANSOUR, J. M. & MOORE, J. J. 2005. Term human fetal membranes have a weak zone overlying the lower uterine pole and cervix before onset of labor. Biol Reprod, 72**,** 720-6.

GRATACOS, E., SANIN-BLAIR, J., LEWI, L., TORAN, N., VERBIST, G., CABERO, L. & DEPREST, J. 2006. A histological study of fetoscopic membrane defects to document membrane healing. Placenta, 27**,** 452-6.

GRATACOS, E., WU, J., YESILDAGLAR, N., DEVLIEGER, R., PIJNENBORG, R. & DEPREST, J. A. 2000. Successful sealing of fetoscopic access sites with collagen plugs in the rabbit model. Am J Obstet Gynecol, 182**,** 142-6.

HARMANLI, O. H., WAPNER, R. J. & LONTZ, J. F. 1998. Efficacy of fibrin glue for in vitro sealing of human chorioamniotic membranes. J Reprod Med, 43**,** 986-90.

JANI, J. C., NICOLAIDES, K. H., GRATACOS, E., VALENCIA, C. M., DONE, E., MARTINEZ, J. M., GUCCIARDO, L., CRUZ, R. & DEPREST, J. A. 2009. Severe

diaphragmatic hernia treated by fetal endoscopic tracheal occlusion. Ultrasound Obstet Gynecol, 34**,** 304-10.

LEE, B. P., DALSIN, J. L. & MESSERSMITH, P. B. 2002. Synthesis and gelation of DOPA-modified poly(ethylene glycol) hydrogels. Biomacromolecules, 3**,** 1038-47.

LEE, H., LEE, B. P. & MESSERSMITH, P. B. 2007. A reversible wet/dry adhesive inspired by mussels and geckos. Nature, 448**,** 338-41.

LIEKENS, D., LEWI, L., JANI, J., HEYNS, L., POLIARD, E., VERBIST, G., OCHSENBEIN-KOLBLE, N., HOYLAERTS, M. & DEPREST, J. 2008. Enrichment of collagen plugs with platelets and amniotic fluid cells increases cell proliferation in sealed iatrogenic membrane defects in the foetal rabbit model. Prenat Diagn, 28**,** 503-7.

LUKS, F. I., DEPREST, J. A., PEERS, K. H., STEEGERS, E. A. & VAN DER WILDT, B. 1999. Gelatin sponge plug to seal fetoscopy port sites: technique in ovine and primate models. Am J Obstet Gynecol, 181**,** 995-6.

MALLIK, A. S., FICHTER, M. A., RIEDER, S., BILIC, G., STERGIOULA, S., HENKE, J., SCHNEIDER, K. T., KURMANAVICIUS, J., BIEMER, E., ZIMMERMANN, R., ZISCH, A. H. & PAPADOPULOS, N. A. 2007. Fetoscopic closure of punctured fetal membranes with acellular human amnion plugs in a rabbit model. Obstet Gynecol, 110**,** 1121-9.

MESSERSMITH, P. B. 2008. Materials science. Multitasking in tissues and materials. Science, 319**,** 1767-8.

OCHSENBEIN-KOLBLE, N., JANI, J., LEWI, L., VERBIST, G., VERCRUYSSE, L., PORTMANN-LANZ, B., MARQUARDT, K., ZIMMERMANN, R. & DEPREST, J. 2007. Enhancing sealing of fetal membrane defects using tissue engineered native amniotic scaffolds in the rabbit model. Am J Obstet Gynecol, 196**,** 263 e1-7.

PAPANNA, R., MOLINA, S., MOISE, K. Y., MOISE, K. J., JR. & JOHNSON, A. 2010. Chorioamnion plugging and the risk of preterm premature rupture of membranes after laser surgery in twin-twin transfusion syndrome. Ultrasound Obstet Gynecol, 35**,** 337-43.

QUINTERO, R. A. 2001. New horizons in the treatment of preterm premature rupture of membranes. Clin Perinatol, 28**,** 861-75.

QUINTERO, R. A., MORALES, W. J., ALLEN, M., BORNICK, P. W., ARROYO, J. & LEPARC, G. 1999. Treatment of iatrogenic previable premature rupture of membranes with intra-amniotic injection of platelets and cryoprecipitate (amniopatch): preliminary experience. Am J Obstet Gynecol, 181**,** 744-9.

QUINTERO, R. A., MORALES, W. J., BORNICK, P. W., ALLEN, M. & GARABELIS, N. 2002. Surgical treatment of spontaneous rupture of membranes: the amniograft--first experience. Am J Obstet Gynecol, 186**,** 155-7.

QUINTERO, R. A., ROMERO, R., DZIECZKOWSKI, J., MAMMEN, E. & EVANS, M. I. 1996. Sealing of ruptured amniotic membranes with intra-amniotic platelet-cryoprecipitate plug. Lancet, 347**,** 1117.

SCISCIONE, A. C., MANLEY, J. S., POLLOCK, M., MAAS, B., SHLOSSMAN, P. A., MULLA, W., LANKIEWICZ, M. & COLMORGEN, G. H. 2001. Intracervical fibrin sealants: a potential treatment for early preterm premature rupture of the membranes. Am J Obstet Gynecol, 184**,** 368-73.

SENAT, M. V., DEPREST, J., BOULVAIN, M., PAUPE, A., WINER, N. & VILLE, Y. 2004. Endoscopic laser surgery versus serial amnioreduction for severe twin-to-twin transfusion syndrome. N Engl J Med, 351**,** 136-44.

SENER, T., OZALP, S., HASSA, H., YALCIN, O. T. & POLAY, S. 1997. Maternal blood clot patch therapy: a model for postamniocentesis amniorrhea. Am J Obstet Gynecol, 177**,** 1535-6.

WAITE, J. H. 1999. Reverse engineering of bioadhesion in marine mussels. Ann N Y Acad Sci, 875**,** 301-9.

WAITE, J. H. & TANZER, M. L. 1980. The bioadhesive of Mytilus byssus: a protein containing L-dopa. Biochem Biophys Res Commun, 96**,** 1554-61.

YAMADA, K., CHEN, T., KUMAR, G., VESNOVSKY, O., TOPOLESKI, L. D. & PAYNE, G. F. 2000. Chitosan based water-resistant adhesive. Analogy to mussel glue. Biomacromolecules, 1**,** 252-8.

YOUNG, B. K., ROMAN, A. S., MACKENZIE, A. P., STEPHENSON, C. D., MINIOR, V., REBARBER, A. & TIMOR-TRITSCH, I. 2004. The closure of iatrogenic membrane defects after amniocentesis and endoscopic intrauterine procedures. Fetal Diagn Ther, 19**,** 296-300.

Figure Legends

FIGURE 1: Biaxial stretching device. (A) The setup consists of a water-filled cylinder where membranes are mounted and inflated with a peristaltic pump. During the inflation, continuous monitoring by a pressure sensor and CCD-cameras is performed. (B) Example of an inflated elastomeric membrane.

FIGURE 2: Deformation behavior of fetal and elastomeric membranes. The distension of elastomeric membranes of 0.5, 1.0 and 2.0mm thickness (n=5) and fetal membranes (n=10) was compared at pressures ranging up to 40mbar by determination of the elevation of the apex (mean ± SD).

FIGURE 3 Biaxial stretching of repaired elastomeric membranes under standardized conditions. (A) Elastomeric membranes of 0.5, 1.0 and 2.0mm thickness were punched to form 3mm defect, which was sealed by a disc shaped (12.9mm diameter, 1.4mm thickness) mussel glue plug. Typical images of inflated elastomeric membranes immediately before rupture, corresponding critical pressures are shown. (B) By increasing the thickness of the elastomeric membranes, the achieved critical pressure increased significantly. The tested groups are all significant in between each other (mean ± SD, * $P \leq 0.05$, ** $P \leq 0.01$).

FIGURE 4: Distension of the mussel glue. (A) Typical images of repaired elastomeric membranes with different thickness are depicted before inflation and at critical pressure. The defect size D and the mussel glue plug size P are indicated. (B) The distension of the defects and the mussel glue plugs, as calculated in percentage of the corresponding initial diameter, were not significantly different between elastomeric membranes with different thicknesses (mean ± SD).

Figure 1a

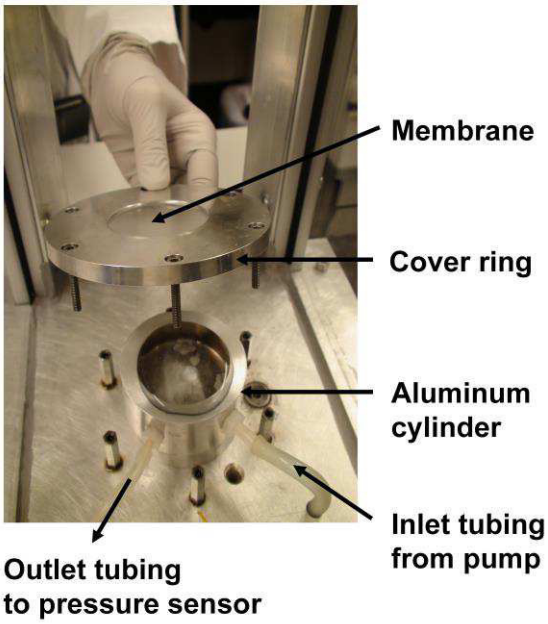


Figure 1b

Figure 2

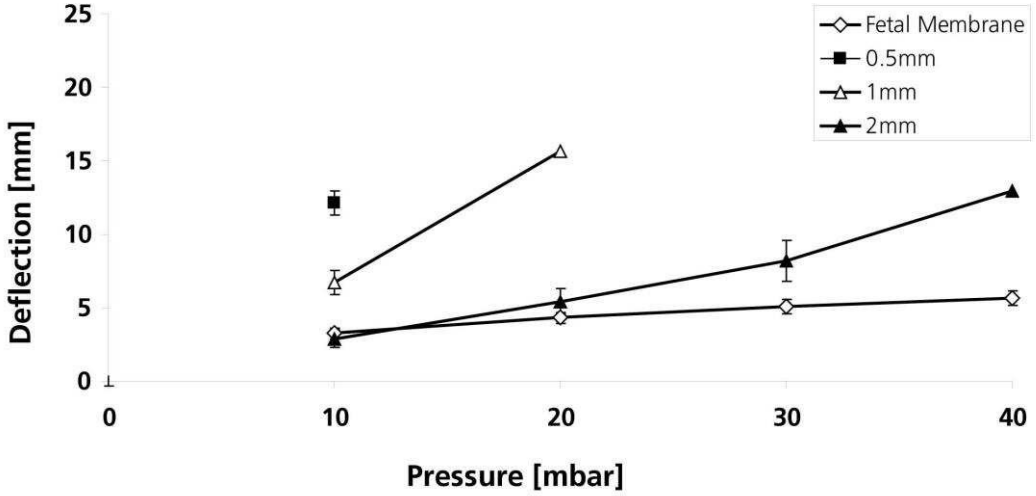

Deflection [mm]
25
20
15
10
5
0
0
10
20
30
40
Pressure [mbar]
Fetal Membrane
0.5mm
1mm
2mm

Figure 3

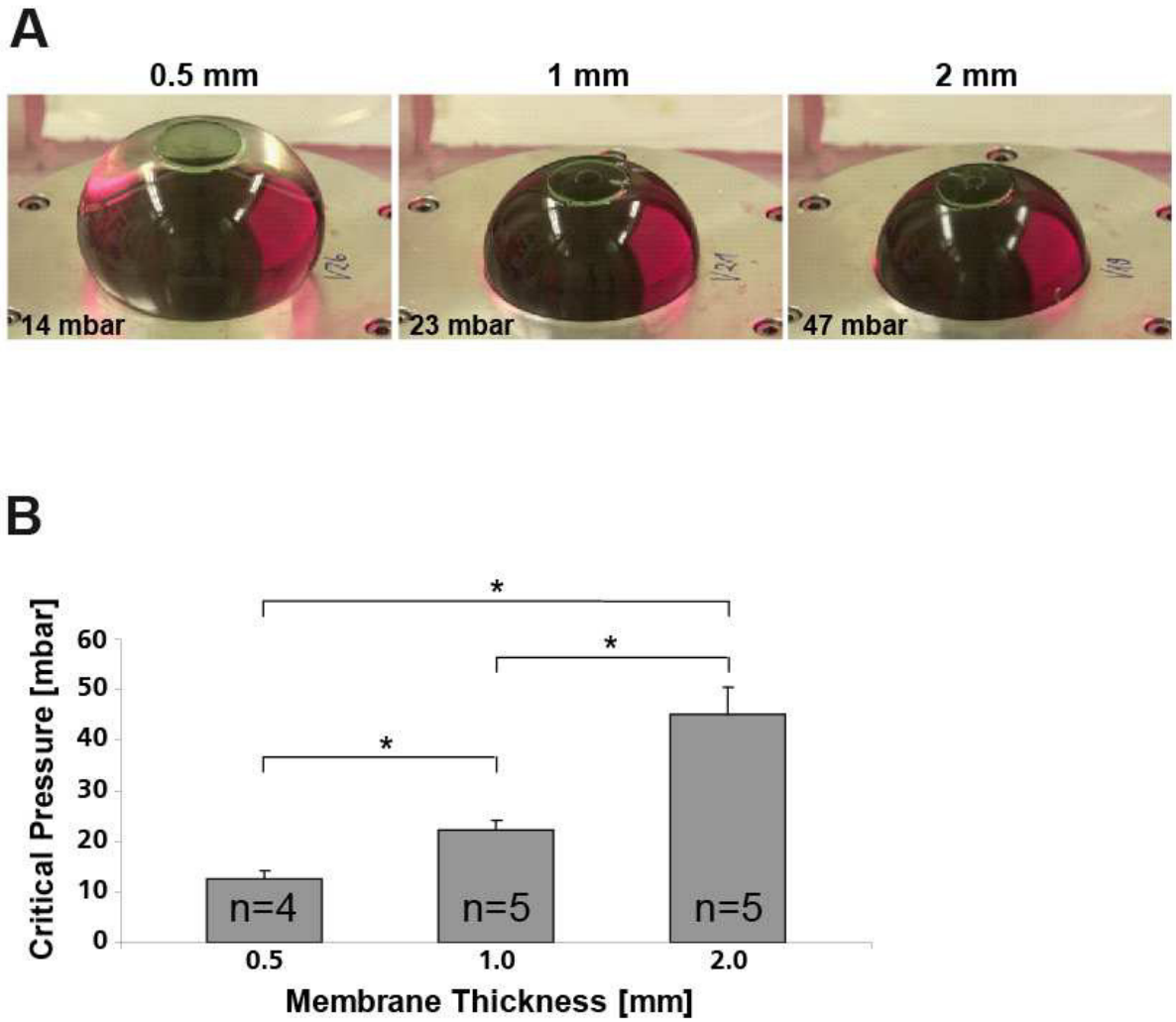
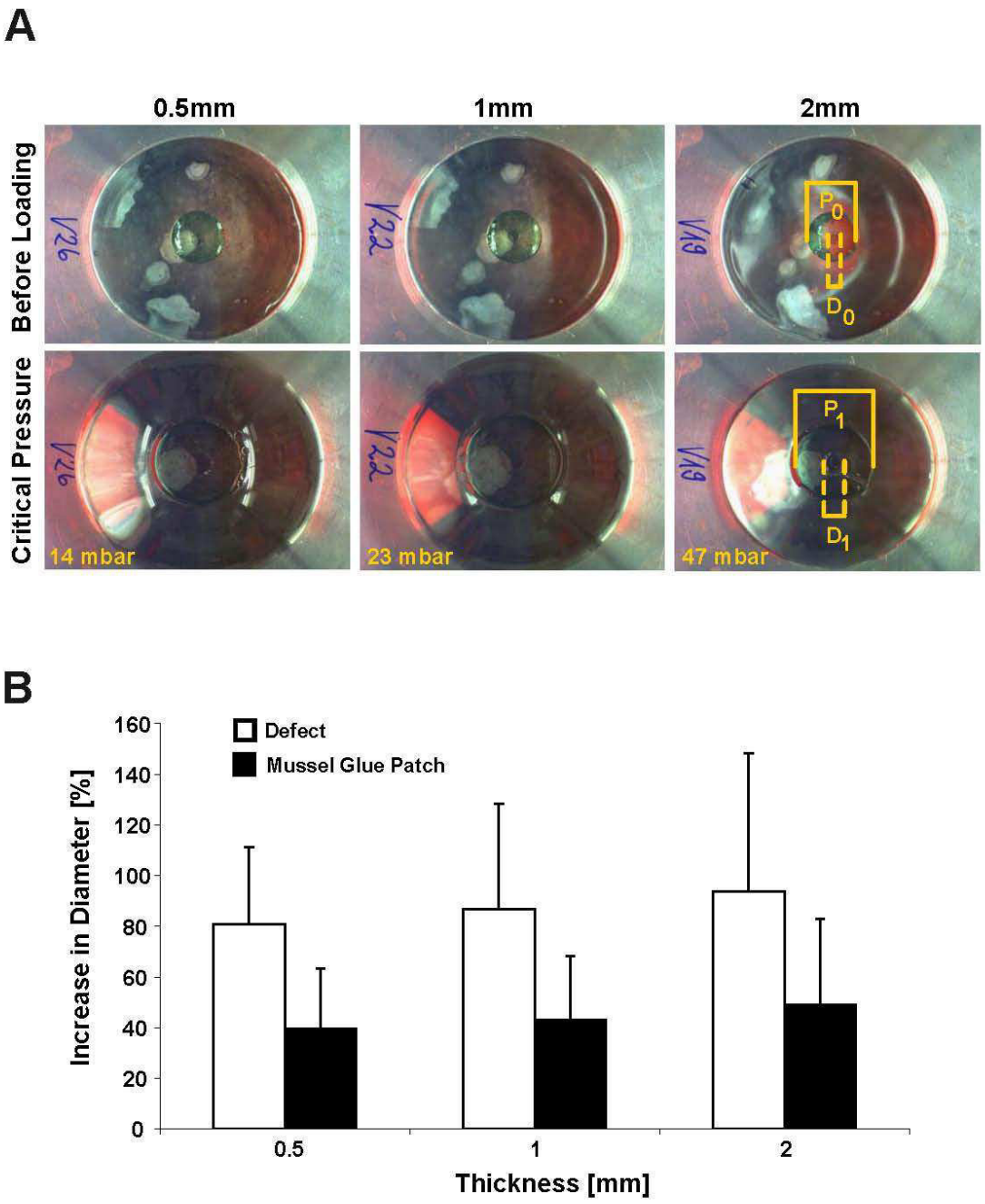

A
0.5 mm
1 mm
2 mm
14 mbar
23 mbar
47 mbar
B
Critical Pressure [mbar]
60
50
40
30
20
10
0
*
*
*
n=4
n=5
n=5
0.5
1.0
2.0
Membrane Thickness [mm]

Figure 4


A
0.5mm
1mm
2mm
Before Loading
Critical Pressure
P0
D0
P1
D1
14 mbar
23 mbar
47 mbar
B
Defect
Mussel Glue Patch
Increase in Diameter [%]
160
140
120
100
80
60
40
20
0
0.5
1
2
Thickness [mm]

2.3. Manuscript III

Mussel-mimetic tissue adhesive for fetal membrane repair: an ex vivo evaluation

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Personal contribution: Design and conduction of all experiments with substantial support in mechanical analysis and characterization of Mussel Glue by Wilfried Bürzle. Writing manuscript.

Abstract

Objective

Iatrogenic preterm prelabour rupture of membranes (iPPROM) remains the main complication after invasive interventions into the intrauterine cavity. Here, the proteolytic stability of mussel-mimetic tissue adhesive (mussel glue) and its sealing behavior on punctured fetal membranes are evaluated.

Study Design

The proteolytic degradation of mussel glue and fibrin glue were compared in vitro. Critical pressures of punctured and sealed fetal membranes were determined under close to physiological conditions using an own-built inflation device. An inverse FE procedure was applied to estimate mechanical parameters of mussel glue.

Results

Mussel glue was insensitive whereas fibrin glue was sensitive towards proteolytic degradation. Mussel glue sealed 3.5 mm fetal membranes defects up to 60 mbar (45 mmHg) when applied under wet conditions, whereas fibrin glue needed dry membrane surfaces for reliable sealing. The mussel glue can be represented by a Neo Hookean material model with elastic coefficient C_1=9.63 kPa.

Conclusions

Ex vivo-tested mussel glue sealed fetal membranes and resisted pressures achieved during uterine contractions. Together with good stability in proteolytic environments, this makes mussel glue a promising sealing material for future applications.

Keywords: Fetal membrane repair; iatrogenic PPROM; membrane sealing; mussel glue

Introduction

With advances in fetal diagnosis the number of performed invasive interventions into the uterine cavity is increasing. Despite improvements in such procedures, even small diameter of fetoscopic access sites do not heal, thus iatrogenic preterm prelabour rupture of fetal membranes (iPPROM) remains the main risk after fetoscopic procedures [1].

During the last decade several strategies to prevent iPPROM have been evaluated. Among such strategies were attempts to stimulate biological repair. Although some repair was observed in rabbit models [2,3] , healing in sheep and rhesus monkey was relatively limited [4,5] . It appears that dislocation of amnion and chorion as well as reattachment of fetal membranes (FM) to the decidua rather than healing leads to sealing of the membrane defects [6]. Due to poor in vivo functionality and stability of materials, prophylactic plugging strategies have not advanced into clinical practice. Major limitations include lack of stable integration of scaffold materials, inability of biocompatible glues to bond to wet surfaces and susceptibility towards proteolytic degradation of naturally derived materials. Such shortcomings of materials generally lead to the instable plugging of the defect and subsequently to leakage shortly after application [7]. As FM are temporary tissues and their repair mechanisms have recently been described to be rather inefficient, the use of gluing materials that are not prone to in vivo remodelling might be envisaged, resulting in sealants that simply act as physical barriers to amniotic fluid.

Recently, star PEG-based polymers have been developed, which are either functionalized with the unusual amino acid 3,4 dihydroxyphenylalanine (DOPA) or catechol-presenting analogues thereof [8,9] . By conversion of catechol group under oxidative conditions, highly reactive quinones are formed [10] that allow strong adherence of the polymer on wet surfaces and gel formation in a saline environment. A catechol functionalized PEG polymer mimic of mussel glue has been employed in a murine model of pancreatic islet transplantation in which

absence of an inflammatory response, long term in vivo stability and good tissue integration were demonstrated [11]. In a recent in vitro study, the same mussel-mimetic tissue adhesive ("mussel glue") has been described to be a non-cytotoxic sealant material that tightly adheres to FM [12].

In order to test its mechanical properties, mussel glue was evaluated in a fully defined in vitro system using elastomeric membranes and a biaxial inflation device [13]. This study demonstrated that mussel glue can be distended and thus adapt to shape changes of the underlying membranes. Although the ductility of the material appeared to be critical for the sealing of compliant membranes, membranes comparable in stiffness to FM were sealed efficiently. As the sealing of FM from such data cannot be extracted, the aim of this study was to evaluate the biochemical stability of mussel glue in vitro and its sealing properties under physiologically relevant conditions ex vivo.

Methods

Mussel glue

The production and characterization of mussel glue, a catechol-functionalized poly(ethylene glycol) (cPEG), which lacks the primary amine of the DOPA amino acid, was performed as described by Brubaker et al. [11]. For the formation of hydrogels, equal volumes of the cPEG precursor solution (300 mg/mL in phosphate-buffered saline (PBS)) and sodium periodate solution (12 mg/mL in water) were mixed and the reaction was allowed to cure for 5 min. at room temperature.

Fibrin sealant

Clinical grade fibrin sealant (TISSEEL, Baxter AG, Vienna) was formed by mixing equal volumes of undiluted fibrin and thrombin solutions using the provided 2-component glue applicator "DUO Set" including the mixing device.

Plug Stability

Polymerization of mussel glue and fibrin glue plugs of equal size was allowed to occur for 5 min. under sterile conditions. The plugs were equilibrated overnight in cell culture medium (DMEM Glutamax F12 + 1 % Penicillin/Streptomycin) at 37 °C and 5 % CO_2 before the weight of each plug was determined and set as 100 %. The gels were daily subjected to fresh cell culture medium which either did or did not contain 8 mU plasmin (Sigma Aldrich, GmbH, Switzerland) or 15 mU collagenase A (Roche, Switzerland). The weight of individual gels was determined on a daily basis and used to determine the relative weight change.

Fetal membrane samples

FM were collected from patients who underwent elective caesarean section between 37 and 38 week of gestation. Patients were recruited for this study with informed written consent using a protocol approved by the Ethical Committee of the District of Zurich (study Stv22/2006). The pregnancies were randomly selected after thorough testing to exclude infections, e.g. HIV, hepatitis, streptococcus B. The selected pregnancies had no history of diabetes, connective tissue disorders, and chromosomal abnormalities. After cutting the FM at least 2 cm away from the placental disc the resulting membrane pieces were washed (PBS, pH 7.2, without Ca/Mg). Round membrane samples of approximately 7 cm diameter were cut out randomly and used within 4 hours.

Design and setup of the inflation device

To assess the sealing properties of mussel mimetic sealant and fibrin sealant, an own-built inflation device was employed that generates an equi-biaxial stress state in the central region of the circular samples. Membrane samples were mounted onto the fluid-filled aluminum cylinder with a 50 mm inner diameter and clamped by a cover ring which was designed to minimize the occurrence of membrane rupture at the sample periphery. The fluid pressure

inside the cylinder was increased by a peristaltic pump (type 314VBM, four rollers, max. 360 rpm, Watson-Marlow Ltd., Zurich, Switzerland), which is computer controlled and allows the inflation of the FM samples. The pressure was constantly recorded by a hydrostatic pressure sensor (Digital manometer, LEX 1, accuracy 0.05 %, Keller, Switzerland). The inflation process was optically monitored by cameras (Point Grey, 1.4MP Color Grasshopper 1394b Camera, 2/3" CCD) mounted on top and on the side of the cylinder.

Fetal Membrane preparation, puncture and repair

FM samples were clamped between two sandpaper rings of 50 mm inner and 70 mm outer diameter to facilitate the mounting procedure. The intact FM were placed such that the amnion was in contact with the fluid in the cylinder and the chorion on the outside. The cover ring was placed onto the water-filled cylinder of our loading device and fixed by a dynamometric screwdriver to reach an equal force of clamping at each screw.

Defects were created in the center of the mounted FM samples using a 16-gauge needle (1.6 mm diameter, Somatex) or a 10-French three-side pointed trocar of 3.5 mm diameter (Richard Wolf GmbH, Knittlingen, Germany). The resulting lesions were directly sealed with 125 µL mussel mimetic or fibrin sealant under wet conditions. For sealing experiments under dry conditions, samples were punctured, carefully dried and sealed before mounting them onto the water-filled cylinder.

Biaxial stretching of fetal membranes

The loading experiment was performed by applying a constant flow of 13.9 mL/min to continuously increase the pressure in the cylinder until rupture of the membrane sample. Rupture was characterized by sudden decrease of internal pressure due to water leakage through a local lesion. The tissue deformation was tracked by the digital images from the side

camera. The membrane profile extracted from each image provided information on the state of deformation associated with the corresponding value of internal pressure.

Histological evaluation of the membrane sealing

Intact and sealed tissue samples were embedded in paraffin and 4 µm cross-sections were cut using the rotation microtome (HM340E, Microtom GmbH, Walldorf, Germany). Haematoxyline & eosine stained histological sections were analyzed with a Zeiss Axiovert 200M microscope (Carl Zeiss, Switzerland)

Mechanical analysis of mussel glue

FMs are inhomogeneous and show a high inter and intra-donor variability. For this reason, a mussel glue mechanical model was based on corresponding observations with inflation experiments on repaired elastomeric membranes [13].

An inverse finite element (FE) procedure was applied to estimate the mechanical parameters of mussel glue. The commercial finite element software package ABAQUS 6.6-3 was used to set up the corresponding axisymmetric model, consisting of the punctured membrane and the glue plug. Geometric and material non-linearities were included. The elastomeric membrane had already been mechanically characterized [14]. The mussel glue was assumed to behave as hyperelastic Neo Hookean material [15].

The inflation experiment was simulated by FE with iterative modification of the material parameters for the glue model to achieve a good agreement between simulated D^{Simu} (Fig. 1a and c) and measured D^{Exp} time histories (Fig. 1b and d) of hole diameter. The optimization problem is automatically solved by the use of the MATLAB function fminsearch [Matlab Documentation, Version 7.10.0 (R2010a), MathWorks, Natick, USA].

Results

Plug stability

In order to determine the relative proteolytic stability, plugs of fibrin and catechol functionalized, 4-arm poly(ethylene glycol) (PEG) based mussel glue (Fig. 2a) were incubated in cell culture medium supplemented with well-defined amounts of collagenase or plasmin. Fibrin glue, as shown by loss of relative plug weight, degraded within 3 days in the presence of collagenase and within 9 days in the presence of plasmin (Fig. 2a). Fibrin sealant in absence of exogenous proteases also degraded within 9 days, indicating the presence of proteolytic enzymes in the material or in the applied cell culture medium. The susceptibility of fibrin sealant towards collagenase and plasmin degradation is in marked contrast to mussel sealant that resists the same conditions over the evaluated period of 22 days without a significant loss in weight (Fig. 2c). Next, materials were exposed to freshly isolated amniotic fluid for a period of 46 days. However, in such experiments only minor weight changes could be observed in fibrin and mussel glue (Fig. 2d).

Deformation during biaxial inflation

To reproduce the injury of amniotic membranes during fetal interventions ex vivo, FM were mounted on the inflation device. The immobilized membranes were then punctured and defects were sealed by the application of sealants in dry or wet conditions. Deformation behaviour of the sealed membranes was observed from side-view imaging. Images taken at the onset of inflation demonstrate that 3.5 mm trocar defects could be functionally sealed under dry and wet conditions by the application of either 125 µL mussel glue or fibrin sealant (Fig. 3a). FM that were sealed with fibrin glue under dry conditions or with mussel glue under wet conditions could be extensively deformed before their rupture (Fig. 3b). In contrast, sealing with fibrin glue under wet conditions appeared to be much less efficient. By careful

evaluation of side-view images, the fibrin sealant plug appeared stiff, initially preventing the deformation of the membranes before lifting off the surface due to weak bonding. The mussel mimetic sealant produces a thin layer on the membranes that adapts its shape with the distension of the membrane and normally fails at the site of the membrane defect (Fig. 3c).

Efficiency of FM sealants

In order to quantify the sealing properties of fibrin sealant and mussel glue under standardized conditions, FM were punctured with a small diameter needle (1.6 mm) or a 10-French three-side pointed trocar (3.5 mm), as would be used during fetal interventions. Small defects could be sealed by both sealants under wet and dry conditions, with achieved mean inflation pressures of 42.3 ± 17.8 mbar (n=4) under wet gluing conditions for mussel glue and 28.1 ± 25.9 mbar (n=3) and 30.3 ± 25.6 mbar (n=3) for fibrin glue under wet or dry sealing conditions, respectively (Fig. 4). However, the large variability between the measured pressures for fibrin glue sealed samples under wet conditions indicates the relatively low reliability of this gluing method. When larger defects were generated, sealing with fibrin glue under wet conditions did not work at all: pressures of only 4.8 ± 6.2 mbar could be measured. In contrast, mussel glue sealed efficiently under wet conditions, allowing inflation to 48.6 ± 18.4 mbar (n = 5). Similar values 47.3 ± 13 mbar (n = 3) could only be achieved by fibrin sealant when membranes were dried before the gluing (Fig. 4).

Histology of sealed fetal membrane samples

The interaction of the tissue sealants with FM was further determined on histological sections. The trocar (3.5 mm) injured and sealed samples were embedded without stretching and tissue cross-sections were microscopically evaluated after haematoxylin and eosin staining (Fig. 5). The sealing materials, mussel glue (dark violet) (Fig. 4b and d) and fibrin sealant (pink) (Fig. 4c and e) were found to tightly interact with the chorion tissue. Both sealants bridge the defect

and might even form a plug within the defect or form an adhesive layer in the vicinity of the defect. Although during histological processing the mussel glue is dislocated from the underlying tissue, cells remain bound to the mussel glue.

Mechanical behaviour of mussel glue

The results of the optimization problem using sealed elastomeric membranes show that the mechanical response of the mussel glue can be represented by a Neo Hookean material model with elastic coefficient C_1=9.63 kPa. The linearized uniaxial response of this model corresponds to a Young's modulus of 57.8 kPa. Data from inflation studies on FM (unpublished data) show that the corresponding linear modulus of fetal membranes is in the range of 111±44 kPa.

Fig. 6 show the comparison of mean stress-strain curve of fetal membranes and mussel glue resulted from FE simulations.

Discussion

In this ex vivo study the favourable proteolytic and biomechanical properties of mussel glue for the sealing of FM are shown and also compared to fibrin glue, a widely used tissue sealant. In contrast to fibrin glue, mussel glue is insensitive towards plasmin and collagenase digestion over a period of 20 days. As the proteases and dosing were clearly not representative of the proteolytic activities present in the amniotic fluid, gluing materials were also exposed to amniotic fluid. To our surprise both fibrin sealant and mussel glue remained stable, with no significant decrease in initial relative weight over a period of 46 days. Since the amniotic fluid undergoes one turn-over within 3 hours the continuous production of proteolytic enzymes might result in a proteolytic environment that cannot be properly studied in vitro [16]. The relative stability of the plugging materials therefore is considered as a good indication for the expected in vivo stability.

Sealing properties of fibrin glue and mussel glue were determined ex vivo on wet membranes to mimic in vivo gluing conditions. Fibrin glue under these conditions sealed small defects, but failed to seal larger defects. Only drying of the membranes resulted in efficient and reproducible sealing. Mussel glue under wet conditions sealed small (1.6 mm) and larger defects (3.5mm) efficiently, reaching values of pressure at rupture up to 60 mbar. These data demonstrate that small membrane defects can be sealed with fibrin or mussel glue under wet conditions, whereas large defects can only be efficiently sealed either under dry conditions or by use of mussel glue. The different behaviour of the two gluing materials might be due to their different chemistries. As fibrin polymerization occurs through aggregation of fibrin monomers to fibrin fibrils, the chemical integration might solely rely on adhesion and occasional factor XIII catalyzed bonds formed between fibrin and extracellular matrix components. On the other hand, mussel glue, with its reactive catechols, is predicted to generate strong covalent and adhesive interactions with the membrane surface. Another

explanation for these findings is differing gelation kinetics and accordingly the changes in viscosity during this process. Fibrin almost instantly forms a viscous solution which solidifies to become a solid plug. The application of the viscous fibrin solution might not allow the complete displacement of the aqueous layer on the surface of the fetal membranes. Therefore, fibrin plug might not be in intimate contact with the membrane, leading to the peeling of the entire plug which was often observed when gluing was performed under wet conditions. Mussel glue in contrast, by its relatively low initial viscosity might be able to displace the aqueous layer and thus form a close contact with the FM before the onset of gelation. In a recently published study, we used mussel glue to seal elastomeric membranes under standardized conditions. Under such conditions the ductility of the material was established as an important parameter for its sealing performance. In this study we have performed a mechanical analysis in order to compare the mechanical behavior of mussel glue with that of FM. The results from the material parameter optimization show that the Young's modulus of the mussel glue is with a value of 57.8 kPa in the same range as the initial uniaxial stiffness of fetal membranes [17]. This indicates the ability of mussel glue to match FM deformation behavior and avoids stress concentrations at the interface between FM and glue, which could lead to a delamination or premature failure. On FM mussel glue withstands pressure of up to 60 mbar (45 mmHg), which is comparable with pressures measured during normal contractions [18] and thus might be sufficient to prevent their preterm rupture in vivo.

This work is limited to ex vivo characterization of mussel glue´s mechanical and proteolytical stability, which in combination with earlier performed in vitro cytotoxicity studies [12] and both, ongoing and published in vivo cytotoxicity studies [11] can partially be used to predict in vivo outcomes. Although, the susceptibility of mussel glue towards proteolytic degradation seems to be rather low, as shown in this study by exposure to prototypical proteases and amniotic fluid, the fate of eventual degradation products need to be further evaluated.

Additionally, membranes derived from the third trimester of pregnancy might have different mechanical and biological properties than membranes in the second trimester, when operative fetoscopic interventions are performed. Thus these data need to be confirmed on second trimester membranes before clinical applications. At this stage our inflation model only simulates FM, neglecting the influence of the uterine wall to the modulation of mechanical forces applied on FM during resting state and contractions in utero. In vivo experiments are to be conducted in appropriate animal models, which allow the testing of sealing efficiency and stability. Finally, strategies and devices need to be developed that allow the fetoscopic application of the gluing material to the site of defect.

Conclusions

This study demonstrates that mussel glue, a fully engineered material, provides superior gluing properties ex vivo than the biologic fibrin glue. These data together with the earlier reported favourable biocompatibility point to mussel glue´s appealing properties for membrane sealing. Such ex vivo data must be reproduced by stability, efficacy, and safety studies in in vivo models.

Acknowledgements

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Disclosure of Interest

None of the authors have a conflict of interest.

References

1 Deprest JA, Flake AW, Gratacos E, Ville Y, Hecher K, Nicolaides K, Johnson MP, Luks FI, Adzick NS, Harrison MR: The making of fetal surgery. Prenat Diagn 2010;30:653-667.

2 Mallik AS, Fichter MA, Rieder S, Bilic G, Stergioula S, Henke J, Schneider KT, Kurmanavicius J, Biemer E, Zimmermann R, Zisch AH, Papadopulos NA: Fetoscopic closure of punctured fetal membranes with acellular human amnion plugs in a rabbit model. Obstet Gynecol 2007;110:1121-1129.

3 Ochsenbein-Kolble N, Jani J, Lewi L, Verbist G, Vercruysse L, Portmann-Lanz B, Marquardt K, Zimmermann R, Deprest J: Enhancing sealing of fetal membrane defects using tissue engineered native amniotic scaffolds in the rabbit model. Am J Obstet Gynecol 2007;196:263 e261-267.

4 Devlieger R, Riley SC, Verbist L, Leask R, Pijnenborg R, Deprest JA: Matrix metalloproteinases-2 and -9 and their endogenous tissue inhibitors in tissue remodeling after sealing of the fetal membranes in a sheep model of fetoscopic surgery. J Soc Gynecol Investig 2002;9:137-145.

5 Devlieger R, Millar LK, Bryant-Greenwood G, Lewi L, Deprest JA: Fetal membrane healing after spontaneous and iatrogenic membrane rupture: A review of current evidence. Am J Obstet Gynecol 2006;195:1512-1520.

6 Gratacos E, Sanin-Blair J, Lewi L, Toran N, Verbist G, Cabero L, Deprest J: A histological study of fetoscopic membrane defects to document membrane healing. Placenta 2006;27:452-456.

7 Quintero RA, Morales WJ, Bornick PW, Allen M, Garabelis N: Surgical treatment of spontaneous rupture of membranes: The amniograft--first experience. Am J Obstet Gynecol 2002;186:155-157.

8 Lee BP, Dalsin JL, Messersmith PB: Synthesis and gelation of dopa-modified poly(ethylene glycol) hydrogels. Biomacromolecules 2002;3:1038-1047.

9 Lee BP, Messersmith PB, Israelachvili JN, Waite JH: Mussel-inspired adhesives and coatings. Annu Rev Mater Res 2011;41:99-132.

10 Lee H, Scherer NF, Messersmith PB: Single-molecule mechanics of mussel adhesion. Proc Natl Acad Sci U S A 2006;103:12999-13003.

11 Brubaker CE, Kissler H, Wang LJ, Kaufman DB, Messersmith PB: Biological performance of mussel-inspired adhesive in extrahepatic islet transplantation. Biomaterials 2010;31:420-427.

12 Bilic G, Brubaker C, Messersmith PB, Mallik AS, Quinn TM, Haller C, Done E, Gucciardo L, Zeisberger SM, Zimmermann R, Deprest J, Zisch AH: Injectable candidate sealants for fetal membrane repair: Bonding and toxicity in vitro. Am J Obstet Gynecol 2010;202:85 e81-89.

13 Haller CM, Buerzle W, Brubaker CE, Messersmith PB, Mazza E, Ochsenbein-Koelble N, Zimmermann R, Ehrbar M: Mussel-mimetic tissue adhesive for fetal membrane repair: A standardized ex vivo evaluation using elastomeric membranes. Prenat Diagn 2011;31:654-660.

14 Wissler MT: Modeling dielectric elastomer actuators. Zurich, Swiss Federal Institute of Technology, 2007, PhD,

15 Moerman KM, Holt CA, Evans SL, Simms CK: Digital image correlation and finite element modelling as a method to determine mechanical properties of human soft tissue in vivo. J Biomech 2009;42:1150-1153.

16 Keith L. Moore TVNP: The developing human: Clinically Oriented Embryology Saunders, 2008, pp 536.

17 Jabareen M, Mallik AS, Bilic G, Zisch AH, Mazza E: Relation between mechanical properties and microstructure of human fetal membranes: An attempt towards a quantitative analysis. Eur J Obstet Gynecol Reprod Biol 2009;144 Suppl 1:S134-141.

18 Maul H, Maner WL, Olson G, Saade GR, Garfield RE: Non-invasive transabdominal uterine electromyography correlates with the strength of intrauterine pressure and is predictive of labor and delivery. J Matern Fetal Neonatal Med 2004;15:297-301.

Figure Legends

FIGURE 1: Axisymmetric finite element (FE) model in reference configuration (a) and in the deformed state (b) as well as corresponding experimental pictures in reference (c) and deformed (d) state. The hole diameter D^{Simu} is analyzed in the FE simulation and compared to the corresponding measurement D^{Exp}. The material parameter of the mussel glue model is iteratively updated to achieve a good agreement between experiment and simulation.

FIGURE 2: Proteolytic stability of tissue glues. (a) Chemical structure of mussel glue. (b) Relative weight change of fibrin glue (n = 3) and (c) mussel glue (n = 3) plugs were determined in vitro in the presence of plasmin (8 mU) and collagenase A (15 mU). (d) Relative weight change of fibrin glue (n = 5) and mussel glue (n = 6) in amniotic fluid. (mean ± SD)

FIGURE 3: Inflation of repaired FM. (a) Side view of the intact or injured and sealed membranes at the onset of inflation (0.8 mbar). (b) Representative images of membranes immediately before rupture show that membranes sealed with mussel glue can be distended to a similar extent than intact membranes. (c) Mussel glue plug ruptures through the site of the sealed defect and fibrin plug lifts off during rupture.

FIGURE 4: Sealing of fetal membranes. (a) FM were punctured with a 1.6 mm needle or a 3.5 mm trocar and sealed with fibrin glue or mussel glue. Mussel glue-sealed membranes resisted high pressures for both defect sizes, whereas fibrin glue sealed larger defects only when applied on dry membranes (mean ± SD). Histology of FM treated with mussel glue (a) on the defect and (c) in the vicinity of the defect or fibrin glue (b) on the defect and (c) in the vicinity of the defect. Haematoxylin and eosin staining show the interaction of the gluing materials mussel glue (violet) and fibrin glue (pink) with the FM.

FIGURE 5: Mechanical characterization of mussel glue. Comparison of the stress–strain curves from the mussel glue with average data of fetal membranes.

Figure 1

Mechanical behaviour of mussel glue.

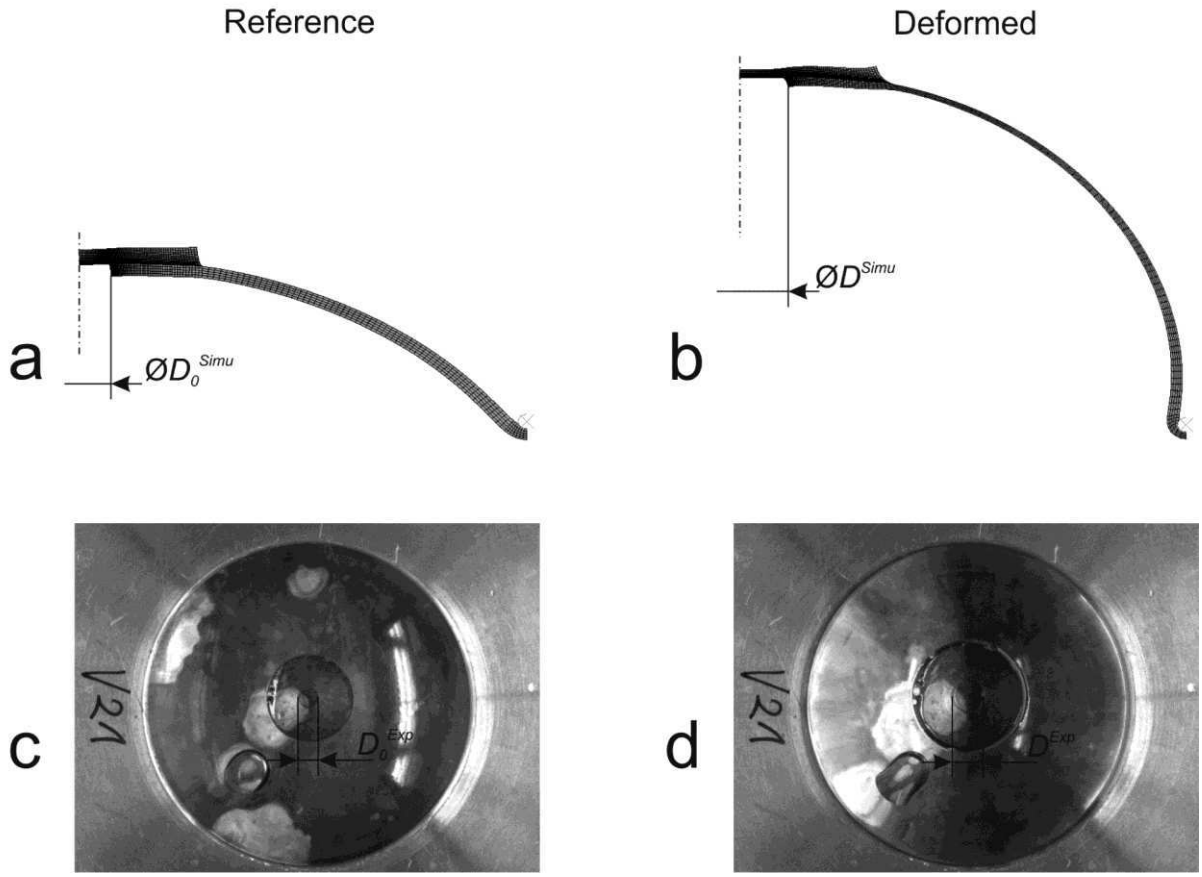
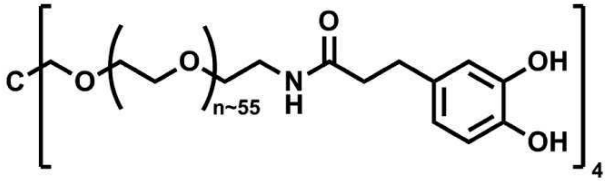


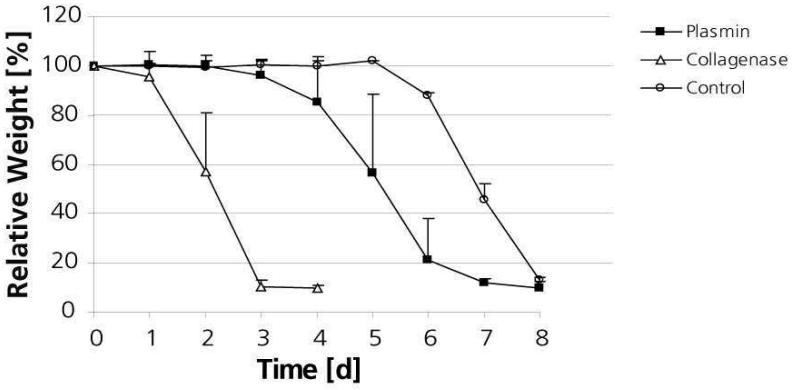
Figure 2

Proteolytic stability of tissue glues.

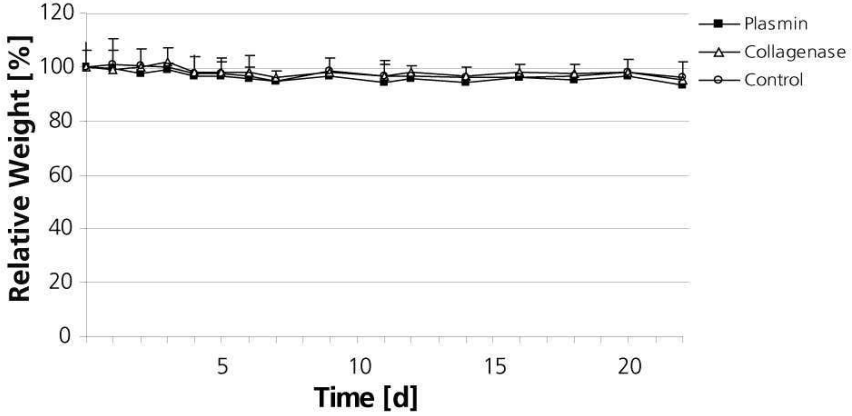
a



b



c



d

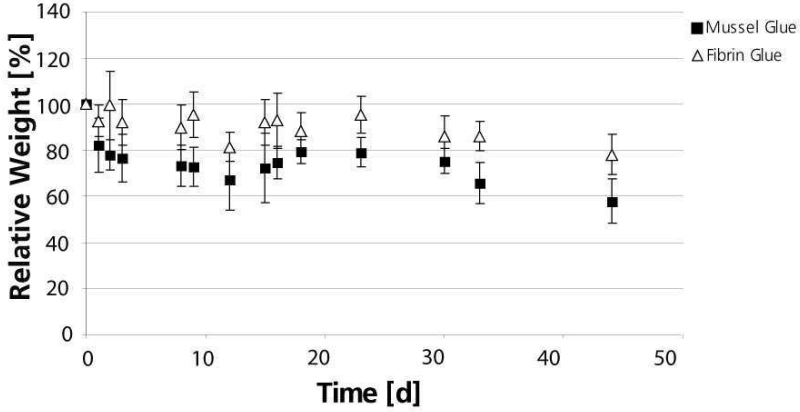


Figure 3

Biaxial stretching of repaired fetal membranes by inflation.

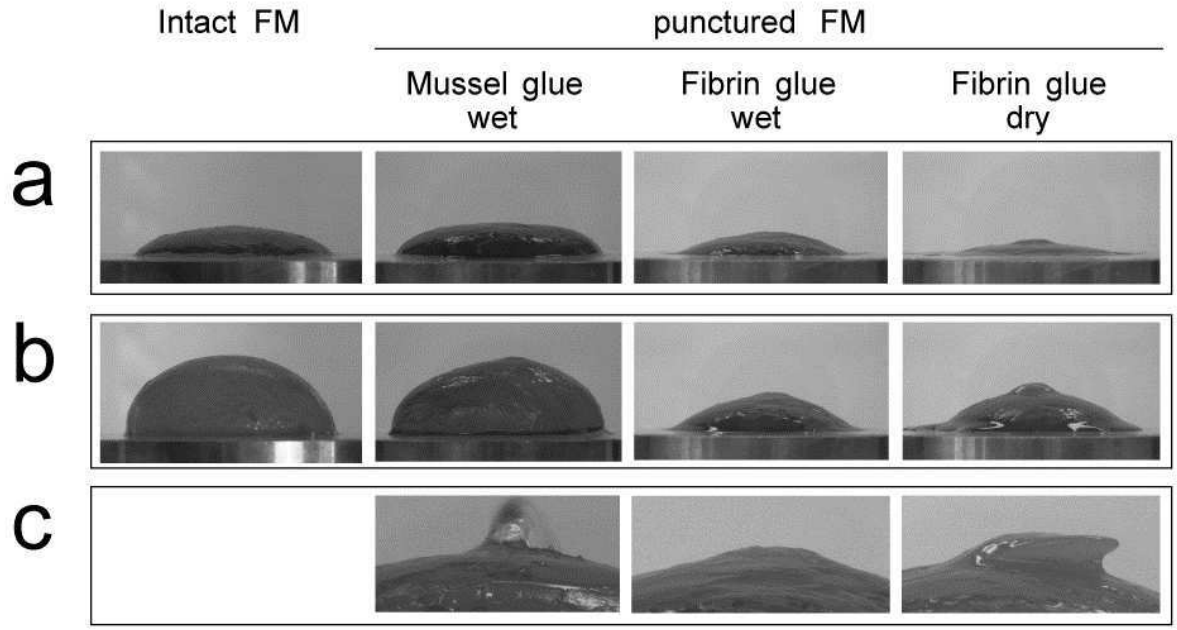
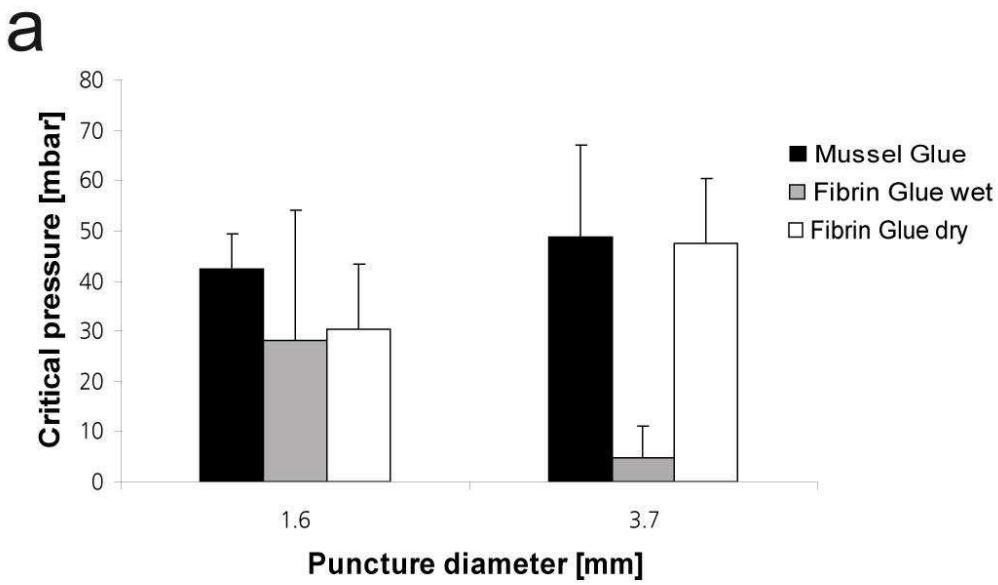


Figure 4

Sealing of fetal membranes.



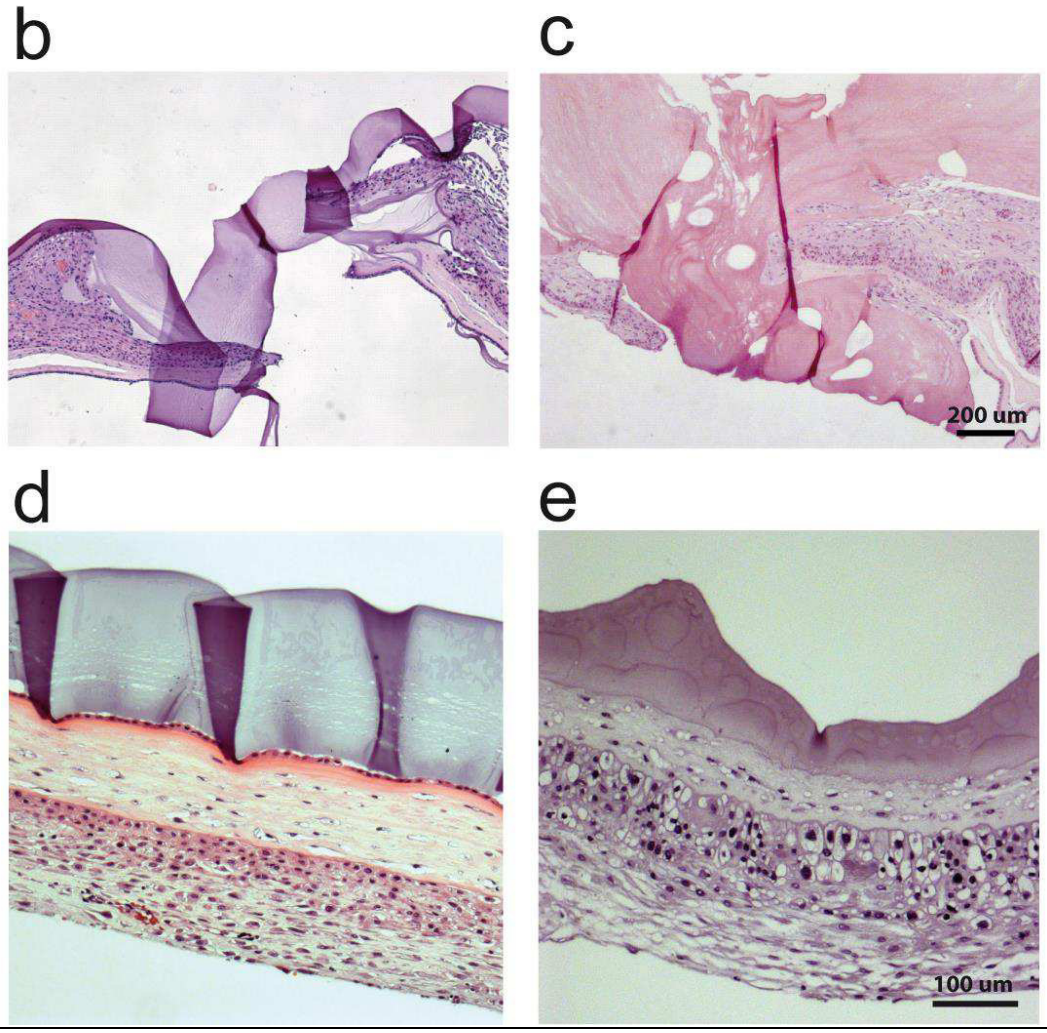
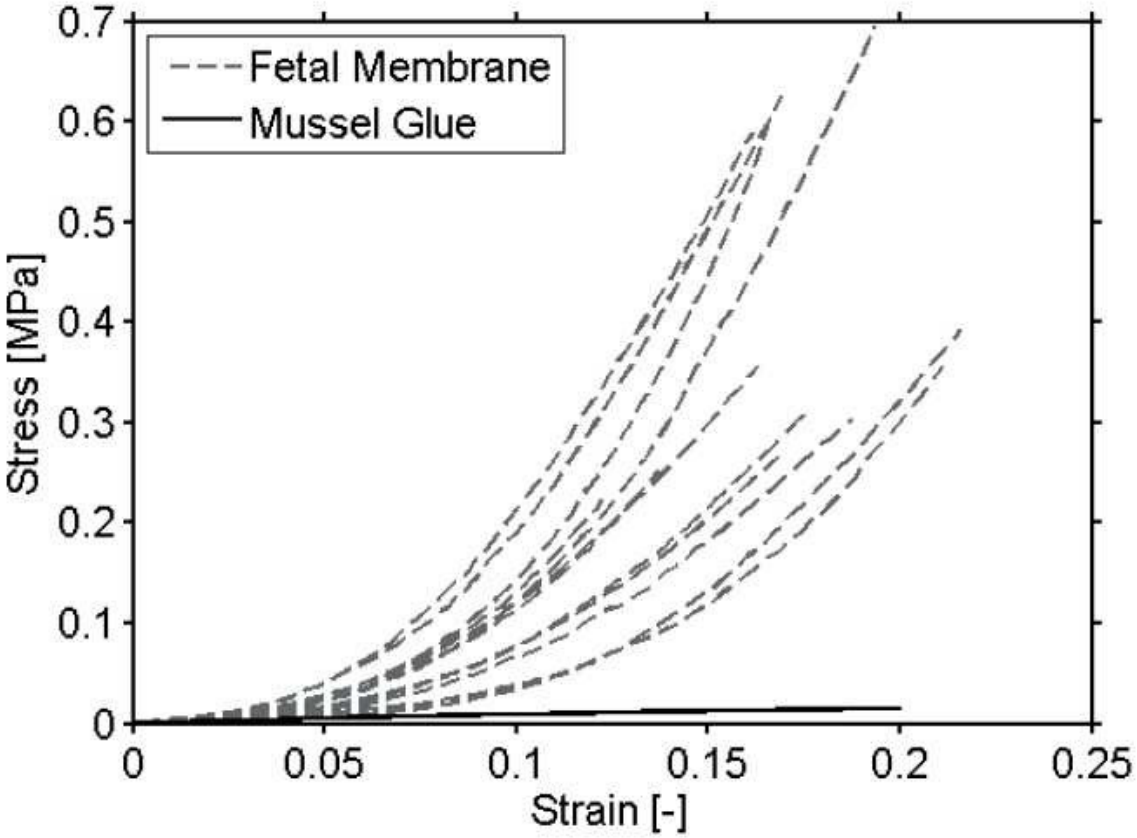


Figure 5


Fetal Membrane
Mussel Glue
Stress [MPa]
Strain [-]
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.05
0.15
0.25

3. General discussion & future perspectives

Iatrogenic PPROM seriously limits the benefit of invasive procedures in the fetal surgery (Deprest, Evrard et al. 1996). Currently, there is no therapy for the treatment of iPPROM routinely employed in hospitals. Therefore, prophylactic measures are seriously considered for clinical application.

In order to develop preventing strategies against fetal membrane rupture from puncture, it is essential to understand the fetal membrane behaviour and the mechanisms leading to membrane failure beforehand. This includes its constituent layers at early gestation, but also near full term, under normal physiological loading conditions. In physiology, the fetal membranes behave in a biaxial state to grow along the pregnancy until rupture at term. Thus, it is necessary to evaluate the biomechanical properties of the membranes in a biaxial state of stress, in order to compare them with the physiological loading conditions. Biaxial inflation best mimics the physiological deformation of the fetal membranes in vivo in terms of loading and rupture condition; although the close simulation is limited to the membrane part overlying the dilated cervix. The remaining parts of the fetal membranes in vivo are in contact with the myometrium and hence demonstrate a different biomechanical situation.

In our first study we applied the biomechanical inflation device that allows biaxial stretching of the fetal membranes to elaborate the fetal membrane's strength properties of amnion and chorion in comparison to the intact fetal membranes. Resulting critical stress data support the opinion that amnion is the load-bearing component, as reported previously (Oxlund, Helmig et al. 1990; Oyen, Cook et al. 2004; Arikat, Novince et al. 2006; Oyen, Calvin et al. 2006), despite being about four times thinner compared to chorion. The presented data indicate the importance of the intact bilayer structure due to higher results in strength values compared to the individual fetal membrane layers. Previous investigators support this indication since the separation of the fetal membrane layers was associated with weakening of the membranes and with the fetal membrane rupture (Strohl, Kumar et al. 2010).

The herein presented intrinsic fetal membrane tension of 0.244 ± 0.093 N/mm is in good agreement with the tension values of 0.205 N/mm reported by Polishuk et al. On the other hand, Joyce et al. found considerably lower membrane tension results of 0.035 N/mm, that were generated using a planar biaxial test system. The comparison of calculated mechanical properties with the corresponding results reported in the literature is not trivial due to the large variability in the experimental setup or in the procedure.

In the reported experiments of intact fetal membranes, the rupture sequence of the layers was observed whenever possible. We detected varying sequences, in some of the membrane samples the failure occurred first in amnion and subsequently chorion ruptured; in other samples the sequence was opposite. For some samples, both of the fetal membrane layers gave up simultaneously at the same location. Beside others, Arikat et al. reported that the chorionic component of the membranes fails first (El Khwad, Stetzer et al. 2005; Arikat, Novince et al. 2006), whereas Schober et al. described that amnion ruptured first (Schober, Kusy et al. 1994; El Khwad, Stetzer et al. 2005). This discrepancy might be explained by the different test methodologies, by the rupture criteria applied as well as by the artefacts which are related to sample fixation. In contrast to our inflation device, the named investigators utilized the biaxial puncture technique. Thereby, local interactions between the plunger and the membrane sample as well as a deformation state in the vicinity of the plunger are generated, which might influence the rupture sequence. Furthermore, in their investigations the failure was related to a reduced puncture force, whereas in our case rupture was identified by the water leakage occurring through local tissue lesions. Just recently, new articles from our group were published presenting data about the rupture sequence of fetal membranes during biomechanical tests on the biaxial inflation device. Results revealed the fracture of amnion before chorion in 73 % of the cases (Buerzle, Haller et al. 2012), 80 % respectively (Haller, Buerzle et al. 2012). The biaxial inflation setup using the fluid increase as the loading force is considered the more representative experiment to simulate the fetal membrane rupture (Buerzle, Haller et al. 2012).

The next part of this thesis describes the correlation of the biomechanical properties of fetal membranes to their microstructural components such as collagen and elastin to gain new insights into the nature and sequence of fetal membrane failure. The attempt was to analyse quantitatively the relationship between the biaxial deformation behaviour and the tissue's microstructure. In a first step we concentrated on the quantity of potentially strength-giving cytoskeletal and ECM proteins such as collagen and elastin. The collagen content identified in amnion resulted in double the amount of chorion, as it was reported in earlier investigations (Halaburt, Uldbjerg et al. 1989; Hampson, Liu et al. 1997; MacDermott and Landon 2000; Meinert, Eriksen et al. 2001). The literature on elastin content differs largely between 0.08 % in fat free dry weight (Hieber, Corcino et al. 1997) and 36 % of wet weight of fresh amnion (Wilshaw et al. 2006). Thus, we are aware that the biochemical data on elastin and collagen are affected by uncertainties related to the great difficulty to quantify collagen and elastin in

tissues. Especially the specificity for elastin in the used biochemical assay should be revised. A trend was observed between biomechanical parameters and biochemical data towards increasing pressure resistance with higher collagen content. Beside a distinct correlation of the collagen amount and the critical membrane tension in amnion, also a correlation between elastin and the mechanical strength was detected in chorion. No correlation was observed between the mechanical strength and the biochemical constituents in the full-thickness FM samples. Nevertheless, we speculate that a membrane tissue with higher amount of elastin and lower amount of collagen behaves more elastic, allowing large deformation with low resistance to high pressures. In this sense the amnion complements the chorion, containing higher collagen content and by this representing a stiffer membrane that withstands high pressures but allows for less deformation due to the lower elastin content. The study of Buerzle et al. confirms that the fetal membranes strength depends on collagen content and the degree of cross-linking (Buerzle, Haller et al. 2012). Cross-links develop between tropocollagens in order to form fibrils and also between fibrils to generate network-like supramolecular structures. Buerzle et al. reported differences concerning the initial stiffness and the critical deformation between uniaxial and biaxial testing which are influenced by the fiber network. A larger compliance of fetal membrane tissue is observed in uniaxial tensile tests compared to biaxial testing where only a low fiber re-orientation, respectively fiber alignment, of collagen was reported (Joyce, Moore et al. 2009). The consequence is a reduced deformation capacity and stiffness. The arrangement of the collagen fiber architecture (i.e. fiber alignment, degree of cross-linking) is crucial to understand and predict the biomechanical properties of the fetal membranes (King, MacDonald et al. 1997). Furthermore, the distribution of the conferred strength and the fashion of its integration into the whole system should be considered. The results of Joyce et al. did highlight the isotropy (i.e. no preferred collagen direction) of intact fetal membranes (Joyce, Moore et al. 2009). These findings are reasonable, because the intact fetal membranes should be able to accommodate all dynamic movements during gestation, and if the fetal membranes get deformed due to fetal movement, the collagen fibers can freely rotate. On the other hand, the same work reported about the amnion layer exhibiting regions of high fiber alignment and being significantly more aligned than the intact membranes and the choriodecidua. These properties are to be evaluated in more detail, so that we can better formulate how the tissue changes in order to fail at full term.

Although there are numerous investigations on the fetal membrane behaviour, substantially more comparable data is needed to obtain accurate knowledge about the nonlinear and viscoelastic nature as well as to understand the baseline failure properties of the fetal membranes. The use of reference points marking the fetal membrane tissue during inflation could provide information not only about apical deflection but also about local 3 - dimensional deformation in order to observe the inhomogeneity within the sample tissue. The examination of the membrane strength, its thickness, biochemical and structural behaviour throughout the entire gestation as well as specification of the sample locations (i.e. relative to the cervix, relative to the rupture site) have to be elaborated in future research. Thickness is an important parameter and essential for the calculation of mechanical parameters. Nevertheless, the thickness measurement is not a simple approach, since the fetal membranes are fibrous tissues with lots of interspaces between fibers and turned out to vary significantly, as also reported by Wittenberg et al. (Wittenberg 2011). By this measurement uncertainty and high variability of the thickness, errors may be introduced in the mechanical calculations.

The so-called “zone of altered morphology” or “weak zone”, which is the natural rupture site above the cervical ostium, has been shown to change towards the end of the pregnancy (Malak and Bell 1994; McLaren, Malak et al. 1999; McLaren, Taylor et al. 2000; McParland, Taylor et al. 2003; El Khwad, Stetzer et al. 2005; El Khwad, Pandey et al. 2006; Moore, Mansour et al. 2006; Moore, Redline et al. 2009). The human bipedal upright posture may lead to higher amount of stretch in the zone overlying the cervical ostium, compared to the remaining fetal membrane regions, due to higher gravitational forces. The relatively high loaded state of the fetal membranes may facilitate its susceptibility to enzymatic degradation, which has been shown to increase with higher stretch (Huang and Yannas 1977). Preliminary experiments were performed separating the potential zone of altered morphology from the remaining membrane parts (data not shown, confirmation needed). Increased structural inhomogeneity and tissue variability may be encountered if such systematically fetal membrane sampling is neglected. Thus, for on-going and future investigations the structural sampling is essential. Previous studies describe a weakening process in the fetal membranes after repeated stretching in vitro (Toppozada, Sallam et al. 1970; Pandey, Jaremko et al. 2007). This somehow simulates the effect of contractions on the fetal membrane rupture which should be analysed in more detail to evaluate if weakening affects mainly the zone of altered morphology or the overall fetal membranes.

Healing of fetal membranes seems to be limited and plugging materials for clinical applications, sealing immediately and over the time period of gestation, have not yet been invented (Devlieger, Gratacos et al. 2000; Gratacos, Sanin-Blair et al. 2006; Liekens, Lewi et al. 2008). Our hypothesis was that glues of biologically inert materials might be fully sufficient for temporary closure of the fetoscopic access sites. Prophylactic plugging intends to apply a plug or sealing right at the time of intervention, just before retrieving the fetoscope. Thus, the next part of the present work aims at repairing human fetal membranes with defined lesions from punctures to reduce the risk for subsequent membrane rupture. In a recent ex vivo study we have shown that mussel glue does not exhibit significant cytotoxic effects on amniotic cells in vitro (Bilic, Brubaker et al. 2010). Additionally, Brubaker et al. showed that mussel glue does not induce any acute or long-term inflammatory response in mice tested for up to one year (Brubaker, Kissler et al. 2010). Mussel glue is a slowly degradable hydrogel indicating excellent adhesion to tissues, that tolerates several times greater shear strength in comparison to fibrin glue (Brubaker and Messersmith 2011). Mussel glue presents appealing characteristics for the application in non-static tissues such as for the occlusion after fetal membrane rupture. Thus, mussel glue's ability to seal and prevent amniotic fluid leakage was evaluated in the present study. As a comparison, fibrin glue, which had been applied for fetal membrane sealing besides many other clinical applications, was tested additionally on its sealing capacity. Fibrin glue was claimed to effectively improve the structural integrity of artificially punctured fetal membranes in vitro (Harmanli, Wapner et al. 1998; Reddy, Shah et al. 2001).

In order to elaborate the mechanical properties of the two glues and test their potential as fetal membrane sealants, a standardized elastomeric membrane was applied as model membranes with well-defined and reproducible material properties. The sealing performance of mussel and fibrin glue upon biaxial inflation was proven on 3 mm defects using standardized procedures. The maximal pressure achieved by the stiffest membrane was determined 51.3 mbar (38.5 mmHg), which is comparable to pre-rupture contractions of about 37 mmHg (Toppozada, Sallam et al. 1970). We suppose that using stiffer model membranes, leading to significantly higher pressures, higher burst pressures could be reached as revealed in (Haller, Buerzle et al. 2011). Elastomeric model membranes exhibit a lower stiffness compared to fresh fetal membranes, at high pressure levels from about 20 mbar onwards. Thus, translating reported results to the stiffer behaving fetal membranes would imply that mussel glue sealed fetal membranes may achieve even higher pressure levels. Furthermore, we demonstrated that

mussel glue is strongly ductile and elastic, as both the defect and the sealing mussel glue plug stretched nearly to their double diameter compared to its origin. A high deformation capacity is a prerequisite for sealants targeted for fetal membrane closure, as fetal membranes grow constantly and are subjected to permanent movement during pregnancy. Moreover, we have noted that the mussel glue plug shape, the plug thickness and the tension distribution greatly influence the sealing performance of the material. Ideally, local stress concentrations at the interface between the glue and fetal membranes are prevented and glue material parameters resemble the fetal membrane's parameter for the deformation behaviour. Nevertheless, this is not very realistic regarding fetal membrane sealing. In order to optimize the gluing performance, these factors will be subject to further investigations and might be challenging for in vivo applications.

In the last part of this thesis, the potential sealing glues were evaluated directly on fresh semi-wet human fetal membranes which were punctured using up to 3.5 mm trocar needles. The sealing capacity of mussel glue was compared to fibrin glue. Mussel glue efficiently sealed fetal membrane defects up to 3.5 mm, resisted to clinically relevant pressure levels (36.5 mmHg) and achieved 10 fold higher pressure levels in contrast to fibrin glue applied under identical conditions. Normal labour amplitudes are in the range between 20 mmHg and 45 mmHg (Maul, Maner et al. 2004), whereas the intrauterine pressure during the second trimester of gestation corresponds to about 5 – 7 mmHg (Sideris and Nicolaides 1990). Fibrin glue demonstrated to seal fetal membrane defects as well if applied under dry conditions, resisting to comparable pressure levels in vivo. Since the intrauterine cavity is an aqueous environment, sealant application under dry conditions in utero would be very challenging. High dilution of glue material strongly reduces the crosslinking potential and may prevent the adhesion and interaction to the tissue. The performed study confirms mussel glue as a potential candidate sealant for the prevention of iPPROM: resistance up to the relevant pressure levels were achieved after the application onto semi-wet fetal membrane tissue. Despite these exciting results, we have no indication how mussel glue sealing does behave under cyclic stretching as well as during a longer timeline. Such investigations will be subject of further in vitro evaluation, wherefore first experiments were conducted in our laboratory. Stable integration, engraftment to the tissue at the defect site and low resorption of plugs is essential for long-lasting closure of fetal membrane defects. In particular, liquid injectible sealants present the crucial effect of strong tissue adhesion; the chance to get displaced from the defect due to movement and contractions is rather low. Mussel glue sealing performs an

elastic and adaptive plaster, covering the defect by a thin layer, strongly adhering to the fetal membranes and forming elastic sealing which permanently adapts to the curvature of the membrane deformation during inflation. Tensile tests performed by others presented excellent tissue adhesion using mussel glue (Brubaker and Messersmith 2011). The adhesion of mussel glue has been found several times stronger in terms of shear strength in comparison to fibrin glue. Mussel glue produces a close contact to the tissue surface expressing covalent bonds which form during the glue polymerization. Essential for the crosslinking and the interfacial bonding is DOPA, a catecholic amino acid derived from tyrosine, incorporated into the PEG polymer and giving rise to the mussel glue precursor. In the presence of periodate, which is a strong oxidant, the catechol group becomes oxidized, also called o-quinone. The strongly reactive DOPA-quinones lead to covalent bonding of primary amines (Lee, Scherer et al. 2006) and thereby to strong interactions between DOPA and organic surfaces. The unoxidized DOPA is capable of performing high-strength and reversible coordination bonds to inorganic surfaces. Brubaker et al. suppose that o-quinone do also react with thiol- and imidazole-groups resulting in a wide interaction with residues available in the ECM proteins of tissues. Such interactions provide a continuous and intact adhesive/tissue interface (Brubaker and Messersmith 2011). We suppose that mussel glue may spread over the defect, the fetal membrane edges as well as the muscular wall and thereby prevent chorioamniotic separation as described for a polymer-based adhesive coacervate inspired by the sandcastle worm (Mann, Papanna et al. 2012). The solidified mussel glue hydrogel is a dense, long-lasting as well as minimally adhesive sealant to nearby tissues (Brubaker, Kissler et al. 2010). These plugging characteristics are clearly favourable in contrast to fibrin glue. Fibrin glue forms rather hard, plug-like shape (more cohesion than adhesion forces), with low adaptation to the fetal membrane's curvature, possibly due to the instant polymerization through aggregation of fibrin monomers to fibrin fibrils. The inflation of plug-like sealed membranes, as observed for fibrin, leads to stress concentration and to detachment of the sealant, presumably due to its higher stiffness or due to the lower adhesion forces to the tissue compared to mussel glue. Nevertheless, fibrin sealant may be successful in vivo due to the supporting myometrium that rests on the fetal membranes and might possibly prevent glue detachment.

In addition, mussel and fibrin glue were tested during 20 days for their in vitro stability in the presence of defined enzymatic activity, which are present in the amniotic fluid among others. As a consequence of the fast (within days to weeks) fibrin glue degradation and difficulties of fibrin glue application in a wet environment, represents no potential sealing candidate for the

prevention of iPPROM. On the other hand, mussel glue was identified as durable glue, without major weight reduction all along the plug incubation. These results line with the long-term stability reported by Brubaker et al., in which work an intact interface of mussel glue with the supporting tissue was maintained for up to one year (Brubaker, Kissler et al. 2010). This formulation, providing an immediate leak-proof and stable sealing, possesses appealing characteristics for the use as a fetal membrane sealant. Nevertheless, these data are clearly preliminary and have to be confirmed by extended exposure of mussel glue to more complex in vivo conditions to establish long-term safety of a potential fetal membrane sealing technique.

In vivo, sealing material can produce intra-amniotic bands as observed in a case with collagen materials, causing a phenomenon that could be compared with an amniotic band syndrome (Devlieger, Ardon et al. 2003). Consequently, amputations and other fetal debilities may develop. We performed preliminary rabbit experiments in the mid-gestational model, in which one amniotic sac was nearly filled with mussel glue, nevertheless the fetus survived with a normal lung to body weight ratio (data not shown). Although this is a single case, no complications were indicated. Nevertheless, during the in vitro sealing of punctured defects by mussel glue, glue parts were observed inside the device cylinder, simulating the “amniotic cavity”, which spread through the defect before polymerizing to generate the sealing. This calls the attention that droplets can possibly enter the cavity during mussel glue application. To resolve whether mussel glue is without risk for the pregnancy, the mother and the fetus, as well as if the ex vivo performance translates into the in vivo situation, it is essential to perform trials in a larger animal series. The full effects of mussel glue as fetal membrane sealing during uterine contractions needs to be evaluated.

The rabbit and the sheep model for the application of preventive plugging of iPPROM in vivo were both introduced by the research team in Leuven, Belgium (Luks, Deprest et al. 1994; Gratacos, Yamamoto et al. 1999). The advantage of the rabbit model is the short gestation time, the large litter size (16 and more rabbit does) and the relatively low expenses for their maintenance. The feasibility of iatrogenic fetal membrane defects, the repair and evaluation of surgical closure techniques can be evaluated using the mid-gestational rabbit model. Several membrane puncture and sealing tests can be performed with one pregnant animal due to the large litter size. Nevertheless, the rabbit model is different to humans and does not totally reflect the human situation, e.g. due to the small size of the model, the late fusion of the amnion with the chorion which in contrast to humans occurs already at around 12 weeks of

gestation, etc. Long-term follow-up studies are impossible in rabbits due to the short gestation time. Due to these facts, the model cannot simulate the human circumstances. The sheep model with litter size of 1 to 3 after spontaneous ovulation in a cycle of 16.5 days and the full gestation time of 145 - 150 days is comparable to humans, but the placentation and feto-maternal interface is different compared to humans (Mossman 1991). The animal model closest to humans is the non-human primate. Rhesus monkeys have, similar to humans, spontaneous ovulation in a cycle of 28 days. Full gestation lasts 165 days and most are singleton pregnancies. Nevertheless, the limited availability, high costs but mostly ethical constraints restrict this model for the evaluation of treatments and techniques which have been proven useful by previous in vitro studies and in vivo experimentation in lower animal species (Ochsenbein-Kölble 2006). To summarize, small animals such as rabbits should be considered to demonstrate the toxicity and feasibility of a potential preventive plugging method, whereas monkeys are necessary to prove anatomic restoration of a defect as well as long-term efficiency of a fetal membrane sealants.

An efficient sealing technique may presents the combination of solid plugs, to achieve an immediate leak proof, combined with the liquid injectable sealing for stable membrane integration. But for the clinical usage, the sealing application should be as simple as possible, e.g. deployment within one step as well as in a short time, and thus this procedure may be too complex for clinical employment. An optimal sealant should further be applicable for already ruptured membranes such as in spontaneous PPROM. It is unclear if mussel glue may be suitable to graft large, undefined defects as they occur upon spontaneous PPROM. In any case, the sealing of fetal membranes after spontaneous PPROM will be difficult, especially due to the unknown defect size and location, the difficult accessibility and so on.

In the presented studies, we focussed on patients undergoing caesarean sections and thus fetal membranes from term pregnancies. Since operative fetoscopy is mostly performed during the second trimester, membranes of early gestation could exhibit different reactivity to sealants as well as different mechanical behaviour. Therefore, additional studies on preterm membranes will be necessary. Further limitations of this present evaluation include the small sample size, the lack of a uterine wall simulation during mechanical experimentation, as well as the missing sealing application through an appropriate trocar used in fetal surgery to access the intrauterine cavity.

In conclusion, the data presented herein demonstrate that mussel glue is the new promising tissue sealant that even binds to semi-wet fetal membrane tissue and can be used as an

efficient injectible sealing material for fetal membrane repair. The present study is limited to ex-vivo characterization of the mussel glue properties. Together with the earlier performed in vitro cytotoxicity study (Bilic et al 2010) and the published in vivo cytotoxicity study (Brubaker et al 2010), this work points out to a new potential membrane sealant with appealing properties. Thus, remain in vivo evaluations to confirm the applicability, efficacy and safety of mussel glue as a prophylactic sealing for fetoscopically punctured fetal membranes and its prevention of iPPROM.

4. References

Al-Zaid, N. S., M. N. Bou-Resli, et al. (1980). "Bursting pressure and collagen content of fetal membranes and their relation to premature rupture of the membranes." Br J Obstet Gynaecol **87**(3): 227-229.

Alger, L. S. and M. J. Pupkin (1986). "Etiology of preterm premature rupture of the membranes." Clin Obstet Gynecol **29**(4): 758-770.

Aplin, J. D., S. Campbell, et al. (1985). "The extracellular matrix of human amniotic epithelium: ultrastructure, composition and deposition." J Cell Sci **79**: 119-136.

Aplin, J. D., S. Campbell, et al. (1986). "Importance of vitamin C in maintenance of the normal amnion: an experimental study." Placenta **7**(5): 377-389.

Arikat, S., R. W. Novince, et al. (2006). "Separation of amnion from choriodecidua is an integral event to the rupture of normal term fetal membranes and constitutes a significant component of the work required." Am J Obstet Gynecol **194**(1): 211-217.

Artal, R., R. E. Burgeson, et al. (1979). "An in vitro model for the study of enzymatically mediated biomechanical changes in the chorioamniotic membranes." Am J Obstet Gynecol **133**(6): 656-659.

Artal, R., R. J. Sokol, et al. (1976). "The mechanical properties of prematurely and non--prematurely ruptured membranes. Methods and preliminary results." Am J Obstet Gynecol **125**(5): 655-659.

Atrah, H. I. (1994). "Fibrin glue." BMJ **308**(6934): 933-934.

Baergen, R. N. (2011). Manual of Pathology of the Human Placenta Boston, Springer Science+Business Media, LLC.

Basaran, M., M. Vural, et al. (2009). "A preliminary study on the use of cyanoacrylate glue for the closure of fetal membranes." Minim Invasive Ther Allied Technol **18**(5): 302-305.

Behzad, F., M. R. Dickinson, et al. (1994). "Brief communication: sliding displacement of amnion and chorion following controlled laser wounding suggests a mechanism for short-term sealing of ruptured membranes." Placenta **15**(7): 775-778.

Bengtson, J. M., L. J. VanMarter, et al. (1989). "Pregnancy outcome after premature rupture of the membranes at or before 26 weeks' gestation." Obstet Gynecol **73**(6): 921-927.

Benirschke K, K. P., Baergen RN (2006). Anatomy and pathology of the placental membranes. In Pathology of the Human Placenta. New York, Springer**:** 321–379.

Benirschke, K. K., P. (1995). Pathology of the human placenta. Berlin, Springer-Verlag.

Bilic, G., C. Brubaker, et al. (2010). "Injectable candidate sealants for fetal membrane repair: bonding and toxicity in vitro." Am J Obstet Gynecol **202**(1): 85 e81-89.

Bourne, G. (1962). "The foetal membranes. A review of the anatomy of normal amnion and chorion and some aspects of their function." Postgrad Med J **38**: 193-201.

Bourne, G. L. (1960). "The microscopic anatomy of the human amnion and chorion." Am J Obstet Gynecol **79**: 1070-1073.

Brubaker, C. E., H. Kissler, et al. (2010). "Biological performance of mussel-inspired adhesive in extrahepatic islet transplantation." Biomaterials **31**(3): 420-427.

Brubaker, C. E. and P. B. Messersmith (2011). "Enzymatically degradable mussel-inspired adhesive hydrogel." Biomacromolecules **12**(12): 4326-4334.

Bryant-Greenwood, G. D. (1998). "The extracellular matrix of the human fetal membranes: structure and function." Placenta **19**(1): 1-11.

Bryant-Greenwood, G. D. and L. K. Millar (2000). "Human fetal membranes: their preterm premature rupture." Biol Reprod **63**(6): 1575-1579.

Buerzle, W., C. M. Haller, et al. (2012). "Multiaxial mechanical behavior of human fetal membranes and its relationship to microstructure." Biomech Model Mechanobiol.

Burke, S. A., M. Ritter-Jones, et al. (2007). "Thermal gelation and tissue adhesion of biomimetic hydrogels." Biomed Mater **2**(4): 203-210.

Calvin, S. E. and M. L. Oyen (2007). "Microstructure and mechanics of the chorioamnion membrane with an emphasis on fracture properties." Ann N Y Acad Sci **1101**: 166-185.

Chang, J., T. F. Tracy, Jr., et al. (2006). "Port insertion and removal techniques to minimize premature rupture of the membranes in endoscopic fetal surgery." J Pediatr Surg **41**(5): 905-909.

Christiansen (1991). The Mechanical Properties of the Extracellular Matrix. Ithaca, NY, USA, Perinatology Press.

Chua, W. K. and M. L. Oyen (2009). "Do we know the strength of the chorioamnion? A critical review and analysis." Eur J Obstet Gynecol Reprod Biol **144 Suppl 1**: S128-133.

Cortes, R. A., A. J. Wagner, et al. (2005). "Pre-emptive placement of a presealant for amniotic access." Am J Obstet Gynecol **193**(3 Pt 2): 1197-1203.

Cox, S. M., M. L. Williams, et al. (1988). "The natural history of preterm ruptured membranes: what to expect of expectant management." Obstet Gynecol **71**(4): 558-562.

Deprest, J. A., V. A. Evrard, et al. (1996). "Endoscopic cord ligation in selective feticide." Lancet **348**(9031): 890-891.

Deprest, J. A., M. G. Munro, et al. (1995). "Review on laparoscopic hysterectomy." Zentralbl Gynakol **117**(12): 641-651.

Devlieger, R., H. Ardon, et al. (2003). "Increased polymorphonuclear infiltration and iatrogenic amniotic band after closure of fetoscopic access sites with a bioactive membrane in the rabbit at midgestation." Am J Obstet Gynecol **188**(3): 844-848.

Devlieger, R., E. Gratacos, et al. (2000). "An organ-culture for in vitro evaluation of fetal membrane healing capacity." Eur J Obstet Gynecol Reprod Biol **92**(1): 145-150.

Devlieger, R., L. K. Millar, et al. (2006). "Fetal membrane healing after spontaneous and iatrogenic membrane rupture: a review of current evidence." Am J Obstet Gynecol **195**(6): 1512-1520.

Dewan, H. and J. M. Morris (2001). "A systematic review of pregnancy outcome following preterm premature rupture of membranes at a previable gestational age." Aust N Z J Obstet Gynaecol **41**(4): 389-394.

Diaz-Zagoya, J. a. F. A. (1980). "In vitro" sealing of ruptured membranes with cryoprecipitate. 27th Annual Meeting of the Society for Gynecologic Investigation. Denver, Colorado.

Draper, D., J. McGregor, et al. (1995). "Elevated protease activities in human amnion and chorion correlate with preterm premature rupture of membranes." Am J Obstet Gynecol **173**(5): 1506-1512.

Duncan, J. M. (1867). "On Cases of Vagina Duplex et Uterus Simplex and of Saccated Uterus." J Anat Physiol **1**(2): 269-274.

El Khwad, M., V. Pandey, et al. (2006). "Fetal membranes from term vaginal deliveries have a zone of weakness exhibiting characteristics of apoptosis and remodeling." J Soc Gynecol Investig **13**(3): 191-195.

El Khwad, M., B. Stetzer, et al. (2005). "Term human fetal membranes have a weak zone overlying the lower uterine pole and cervix before onset of labor." Biol Reprod **72**(3): 720-726.

Ernst, L. M. (2011). Color atlas of fetal and neonatal histology. New york, Springer Science+Business Media, LLC.

Fawthrop, R. K. and C. D. Ockleford (1994). "Cryofracture of human term amniochorion." Cell Tissue Res **277**(2): 315-323.

Fortunato, S. J., R. Menon, et al. (1997). "Collagenolytic enzymes (gelatinases) and their inhibitors in human amniochorionic membrane." Am J Obstet Gynecol **177**(4): 731-741.

Genz, H. J. (1979). "[Treatment of premature rupture of the fetal membranes by means of fibrin adhesion]." Med Welt **30**(42): 1557-1559.

Goldenberg, R. L., J. F. Culhane, et al. (2008). "Epidemiology and causes of preterm birth." Lancet **371**(9606): 75-84.

Gratacos, E., J. Sanin-Blair, et al. (2006). "A histological study of fetoscopic membrane defects to document membrane healing." Placenta **27**(4-5): 452-456.

Gratacos, E., J. Wu, et al. (2000). "Successful sealing of fetoscopic access sites with collagen plugs in the rabbit model." Am J Obstet Gynecol **182**(1 Pt 1): 142-146.

Gratacos, E., H. Yamamoto, et al. (1999). "The midgestational rabbit as a model for the creation of membrane defects after needle fetoscopy." Am J Obstet Gynecol **180**(5): 1263-1267.

Halaburt, J. T., N. Uldbjerg, et al. (1989). "The concentration of collagen and the collagenolytic activity in the amnion and the chorion." Eur J Obstet Gynecol Reprod Biol **31**(1): 75-82.

Haller, C. M., W. Buerzle, et al. (2011). "Mussel-mimetic tissue adhesive for fetal membrane repair: a standardized ex vivo evaluation using elastomeric membranes." Prenat Diagn **31**(7): 654-660.

Haller, C. M., W. Buerzle, et al. (2012). "Mussel-mimetic tissue adhesive for fetal membrane repair: an ex vivo evaluation." Acta Biomater **8**(12): 4365-4370.

Hampson, V., D. Liu, et al. (1997). "Amniotic membrane collagen content and type distribution in women with preterm premature rupture of the membranes in pregnancy." Br J Obstet Gynaecol **104**(9): 1087-1091.

Harmanli, O. H., R. J. Wapner, et al. (1998). "Efficacy of fibrin glue for in vitro sealing of human chorioamniotic membranes." J Reprod Med **43**(11): 986-990.

Harrison, M. R., R. L. Keller, et al. (2003). "A randomized trial of fetal endoscopic tracheal occlusion for severe fetal congenital diaphragmatic hernia." N Engl J Med **349**(20): 1916-1924.

Health, E. o. Mai 2011, from http://www.beltina.org/pics/amniotic_fluid.jpg.

Helmig, R., H. Oxlund, et al. (1993). "Different biomechanical properties of human fetal membranes obtained before and after delivery." Eur J Obstet Gynecol Reprod Biol **48**(3): 183-189.

Hempel, E. (1972). "[Ultrastructural differentiation of human amnion epithelium with special reference to the umbilical cord]." Anat Anz **132**(3): 356-370.

Hertig, A. T., J. Rock, et al. (1956). "A description of 34 human ova within the first 17 days of development." Am J Anat **98**(3): 435-493.

Hieber, A. D., D. Corcino, et al. (1997). "Detection of elastin in the human fetal membranes: proposed molecular basis for elasticity." Placenta **18**(4): 301-312.

Hofmeyr, G. J., G. Essilfie-Appiah, et al. (2011). "Amnioinfusion for preterm premature rupture of membranes." Cochrane Database Syst Rev **12**: CD000942.

Huang, C. and I. V. Yannas (1977). "Mechanochemical studies of enzymatic degradation of insoluble collagen fibers." J Biomed Mater Res **11**(1): 137-154.

Instruments, J. (2009). "Observing the Levels of Alignment and Organisation in Collagen Structures Using Atomic Force Microscopy by JPK Instruments." Retrieved July 2011, 2011, from http://www.azonano.com/images/Article_Images/ImageForArticle_2267(2).jpg.

Jabareen, M., A. S. Mallik, et al. (2009). "Relation between mechanical properties and microstructure of human fetal membranes: an attempt towards a quantitative analysis." Eur J Obstet Gynecol Reprod Biol **144 Suppl 1**: S134-141.

Jain, V. D. and A. Sciscione (2011). "Considerations in membrane resealing after preterm PROM." Clin Obstet Gynecol **54**(2): 351-357.

Johnson, J. W., R. S. Egerman, et al. (1990). "Cases with ruptured membranes that "reseal"." Am J Obstet Gynecol **163**(3): 1024-1030; discussion 1030-1022.

Joyce, E. M., J. J. Moore, et al. (2009). "Biomechanics of the fetal membrane prior to mechanical failure: review and implications." Eur J Obstet Gynecol Reprod Biol **144 Suppl 1**: S121-127.

Kanayama, N., T. Terao, et al. (1985). "Collagen types in normal and prematurely ruptured amniotic membranes." Am J Obstet Gynecol **153**(8): 899-903.

Keirse MJNC, O. A., Treffers PE, Kanhai HHH (1989). Prelabour rupture of the membranes preterm. Effective Care in Pregnancy and Childbirth. E. M. Chalmers I, Keirse MJNC Oxford, : Oxford University Press.

Keith L. Moore, T. V. N. p. (2008). The Developing Human, Saunders.

Kielty, C. M., M. J. Sherratt, et al. (2002). "Elastic fibres." J Cell Sci **115**(Pt 14): 2817-2828.

Kielty, C. M. a. G., M.E. (2002). The collagen family: structure, assembly, and organization in the extracellular matrix. Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects. P. M. R. a. Steinmann. New York, Wiley-Liss**:** 159-221.

King, L. A., P. C. MacDonald, et al. (1997). "Regulation of metallothionein expression in human amnion epithelial and mesenchymal cells." Am J Obstet Gynecol **177**(6): 1496-1501.

Lavery, J. P. and C. E. Miller (1977). "The viscoelastic nature of chorioamniotic membranes." Obstet Gynecol **50**(4): 467-472.

Lavery, J. P. and C. E. Miller (1979). "Deformation and creep in the human chorioamniotic sac." Am J Obstet Gynecol **134**(4): 366-375.

Lavery, J. P., C. E. Miller, et al. (1980). "Effect of meconium on the strength of chorioamniotic membranes." Obstet Gynecol **56**(6): 711-715.

Lavery, J. P., C. E. Miller, et al. (1982). "The effect of labor on the rheologic response of chorioamniotic membranes." Obstet Gynecol **60**(1): 87-92.

Lee, B. P., J. L. Dalsin, et al. (2002). "Synthesis and gelation of DOPA-modified poly(ethylene glycol) hydrogels." Biomacromolecules **3**(5): 1038-1047.

Lee, H., S. M. Dellatore, et al. (2007). "Mussel-inspired surface chemistry for multifunctional coatings." Science **318**(5849): 426-430.

Lee, H., B. P. Lee, et al. (2007). "A reversible wet/dry adhesive inspired by mussels and geckos." Nature **448**(7151): 338-341.

Lee, H., N. F. Scherer, et al. (2006). "Single-molecule mechanics of mussel adhesion." Proc Natl Acad Sci U S A **103**(35): 12999-13003.

Lei, H., E. E. Furth, et al. (1996). "A program of cell death and extracellular matrix degradation is activated in the amnion before the onset of labor." J Clin Invest **98**(9): 1971-1978.

Leppert, P. C. and S. Y. Yu (1991). "Three-dimensional structures of uterine elastic fibers: scanning electron microscopic studies." Connect Tissue Res **27**(1): 15-31.

Leushner, J. R. and C. L. Clarson (1986). "Analysis of the collagens from the fetal membranes of diabetic mothers." Placenta **7**(1): 65-72.

Lewi, L., D. Van Schoubroeck, et al. (2004). "Successful patching of iatrogenic rupture of the fetal membranes." Placenta **25**(4): 352-356.

Li, S., D. Harrison, et al. (2002). "Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation." J Cell Biol **157**(7): 1279-1290.

Liekens, D., L. Lewi, et al. (2008). "Enrichment of collagen plugs with platelets and amniotic fluid cells increases cell proliferation in sealed iatrogenic membrane defects in the foetal rabbit model." Prenat Diagn **28**(6): 503-507.

Lockwood, C. J., A. E. Senyei, et al. (1991). "Fetal fibronectin in cervical and vaginal secretions as a predictor of preterm delivery." N Engl J Med **325**(10): 669-674.

Louis-Sylvestre, C., P. Morice, et al. (1997). "The role of laparoscopy in the diagnosis and management of heterotopic pregnancies." Hum Reprod **12**(5): 1100-1102.

Luks, F. I., J. A. Deprest, et al. (1999). "Gelatin sponge plug to seal fetoscopy port sites: technique in ovine and primate models." Am J Obstet Gynecol **181**(4): 995-996.

Luks, F. I., J. A. Deprest, et al. (1994). "A model for fetal surgery through intrauterine endoscopy." J Pediatr Surg **29**(8): 1007-1009.

MacDermott, R. I. J. and C. R. Landon (2000). "The hydroxyproline content of amnion and prelabour rupture of the membranes." European Journal of Obstetrics Gynecology and Reproductive Biology **92**(2): 217-221.

Maclachlan, T. B. (1965). "A Method for the Investigation of the Strength of the Fetal Membranes." Am J Obstet Gynecol **91**: 309-313.

Malak, T. M. and S. C. Bell (1994). "Structural characteristics of term human fetal membranes: a novel zone of extreme morphological alteration within the rupture site." Br J Obstet Gynaecol **101**(5): 375-386.

Malak, T. M., C. D. Ockleford, et al. (1993). "Confocal immunofluorescence localization of collagen types I, III, IV, V and VI and their ultrastructural organization in term human fetal membranes." Placenta **14**(4): 385-406.

Malak, T. M., F. Sizmur, et al. (1996). "Fetal fibronectin in cervicovaginal secretions as a predictor of preterm birth." Br J Obstet Gynaecol **103**(7): 648-653.

Mallik, A. S., M. A. Fichter, et al. (2007). "Fetoscopic closure of punctured fetal membranes with acellular human amnion plugs in a rabbit model." Obstet Gynecol **110**(5): 1121-1129.

Mann, L. K., R. Papanna, et al. (2012). "Fetal membrane patch and biomimetic adhesive coacervates as a sealant for fetoscopic defects." Acta Biomater **8**(6): 2160-2165.

Maul, H., W. L. Maner, et al. (2004). "Non-invasive transabdominal uterine electromyography correlates with the strength of intrauterine pressure and is predictive of labor and delivery." J Matern Fetal Neonatal Med **15**(5): 297-301.

McLaren, J., T. M. Malak, et al. (1999). "Structural characteristics of term human fetal membranes prior to labour: identification of an area of altered morphology overlying the cervix." Hum Reprod **14**(1): 237-241.

McLaren, J., D. J. Taylor, et al. (2000). "Increased concentration of pro-matrix metalloproteinase 9 in term fetal membranes overlying the cervix before labor: implications for membrane remodeling and rupture." Am J Obstet Gynecol **182**(2): 409-416.

McParland, P. C., D. J. Taylor, et al. (2003). "Mapping of zones of altered morphology and chorionic connective tissue cellular phenotype in human fetal membranes (amniochorion and decidua) overlying the lower uterine pole and cervix before labor at term." Am J Obstet Gynecol **189**(5): 1481-1488.

Mehats, C., T. Schmitz, et al. (2011). "[Biochemistry of fetal membranes rupture]." Gynecol Obstet Fertil **39**(6): 365-369.

Meinert, M., G. V. Eriksen, et al. (2001). "Proteoglycans and hyaluronan in human fetal membranes." Am J Obstet Gynecol **184**(4): 679-685.

Meinert, M., A. Malmstrom, et al. (2007). "Labour induces increased concentrations of biglycan and hyaluronan in human fetal membranes." Placenta **28**(5-6): 482-486.

Menon, R. and S. J. Fortunato (2007). "Infection and the role of inflammation in preterm premature rupture of the membranes." Best Pract Res Clin Obstet Gynaecol **21**(3): 467-478.

Mercer, B. M. (2003). "Preterm premature rupture of the membranes." Obstet Gynecol **101**(1): 178-193.

Mercer, B. M., R. L. Goldenberg, et al. (1999). "The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National

Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network." Am J Obstet Gynecol **181**(5 Pt 1): 1216-1221.

Moore, R. M., J. M. Mansour, et al. (2006). "The physiology of fetal membrane rupture: insight gained from the determination of physical properties." Placenta **27**(11-12): 1037-1051.

Moore, R. M., R. W. Redline, et al. (2009). "Differential expression of fibulin family proteins in the para-cervical weak zone and other areas of human fetal membranes." Placenta **30**(4): 335-341.

Mossman, H. W. (1991). "Classics revisited: Comparative morphogenesis of the fetal membranes and accessory uterine structures." Placenta **12**(1): 1-5.

Naeye, R. L. (1982). "Factors that predispose to premature rupture of the fetal membranes." Obstet Gynecol **60**(1): 93-98.

Ochsenbein-Kölble, N. (2006). Human fetal membranes and new therapeutic strategies for preterm prelabour rupture of the membranes - from amniotic cells to scaffolds. Zürich, Switzerland, University Zürich**:** 33.

Ochsenbein-Kolble, N., J. Jani, et al. (2007). "Enhancing sealing of fetal membrane defects using tissue engineered native amniotic scaffolds in the rabbit model." Am J Obstet Gynecol **196**(3): 263 e261-267.

Ockleford, C., N. Bright, et al. (1993). "Micro-trabeculae, macro-plaques or mini-basement membranes in human term fetal membranes?" Philos Trans R Soc Lond B Biol Sci **342**(1300): 121-136.

Osman, I., A. Young, et al. (2006). "Leukocyte density and proinflammatory mediator expression in regional human fetal membranes and decidua before and during labor at term." J Soc Gynecol Investig **13**(2): 97-103.

Oxlund, H., R. Helmig, et al. (1990). "Biomechanical analysis of human chorioamniotic membranes." Eur J Obstet Gynecol Reprod Biol **34**(3): 247-255.

Oyen, M. L., S. E. Calvin, et al. (2006). "Premature rupture of the fetal membranes: is the amnion the major determinant?" Am J Obstet Gynecol **195**(2): 510-515.

Oyen, M. L., R. F. Cook, et al. (2004). "Mechanical failure of human fetal membrane tissues." J Mater Sci Mater Med **15**(6): 651-658.

Pandey, V., K. Jaremko, et al. (2007). "The force required to rupture fetal membranes paradoxically increases with acute in vitro repeated stretching." Am J Obstet Gynecol **196**(2): 165 e161-167.

Papadopulos, N. A., P. P. Van Ballaer, et al. (1998). "Fetal membrane closure techniques after hysteroamniotomy in the midgestational rabbit model." Am J Obstet Gynecol **178**(5): 938-942.

Parry-Jones, E. and S. Priya (1976). "A study of the elasticity and tension of fetal membranes and of the relation of the area of the gestational sac to the area of the uterine cavity." Br J Obstet Gynaecol **83**(3): 205-212.

Parry, S. and J. F. Strauss, 3rd (1998). "Premature rupture of the fetal membranes." N Engl J Med **338**(10): 663-670.

Patrick, J., K. Campbell, et al. (1982). "Patterns of gross fetal body movements over 24-hour observation intervals during the last 10 weeks of pregnancy." Am J Obstet Gynecol **142**(4): 363-371.

Phupong, V. and P. Ultchaswadi (2006). "Spontaneous reseal of ruptured membranes after genetic amniocentesis." J Med Assoc Thai **89**(7): 1033-1035.

Polishuk, W. Z., S. Kohane, et al. (1962). "The physical properties of fetal membranes." Obstet Gynecol **20**: 204-210.

Polzin, W. J. and K. Brady (1991). "Mechanical factors in the etiology of premature rupture of the membranes." Clin Obstet Gynecol **34**(4): 702-714.

Pressman, E. K., J. L. Cavanaugh, et al. (2002). "Physical properties of the chorioamnion throughout gestation." Am J Obstet Gynecol **187**(3): 672-675.

Prévost, T. P. (2006). Biomechanics of the Human Chorioamnion. Department of Materials Science and Engineering, Massachusetts Institute of Technology. **Master of Science:** 115.

Quintero, R. A., W. J. Morales, et al. (1999). "Treatment of iatrogenic previable premature rupture of membranes with intra-amniotic injection of platelets and cryoprecipitate (amniopatch): preliminary experience." Am J Obstet Gynecol **181**(3): 744-749.

Quintero, R. A., W. J. Morales, et al. (2002). "Surgical treatment of spontaneous rupture of membranes: the amniograft--first experience." Am J Obstet Gynecol **186**(1): 155-157.

Quintero, R. A., R. Romero, et al. (1996). "Sealing of ruptured amniotic membranes with intra-amniotic platelet-cryoprecipitate plug." Lancet **347**(9008): 1117.

R, H. (1990). Fibronectins. New York, Springer.

R. Lanza, R. L. a. J. V. (2007). Principles of Tissue Engineering, Elsevier**:** 101-115.

Reddy, U. M., S. S. Shah, et al. (2001). "In vitro sealing of punctured fetal membranes: potential treatment for midtrimester premature rupture of membranes." Am J Obstet Gynecol **185**(5): 1090-1093.

Reti, N. G., M. Lappas, et al. (2007). "Why do membranes rupture at term? Evidence of increased cellular apoptosis in the supracervical fetal membranes." Am J Obstet Gynecol **196**(5): 484 e481-410.

Rotschild, A., E. W. Ling, et al. (1990). "Neonatal outcome after prolonged preterm rupture of the membranes." Am J Obstet Gynecol **162**(1): 46-52.

Sadler, T. W., Langman, Jan (2009). Langman's medical embryology Baltimore, Wolters Kluwer Lippincott William & Wilkins.

Schmidt, W. (1963). "[Structure and Function of Amnion Epithelium in Man and Fowl]." Z Zellforsch Mikrosk Anat **61**: 642-660.

Schmidt, W. (1992). The Amniotic Fluid Compartement: The Fetal Habitat.

Schober, E. A., R. P. Kusy, et al. (1994). "Resistance of fetal membranes to concentrated force applications and reconciliation of puncture and burst testing." Ann Biomed Eng **22**(5): 540-548.

Schoenwolf, G. C. e. a. (2009). Larsen's human embryology Philadelphia, Churchill Livingstone.

Schwarzfurtner, H., I. Huttegger, et al. (1986). "[Quantitative studies of the collagen content of the human amnion and chorion]." Anat Anz **161**(1): 3-9.

Sciscione, A. C., J. S. Manley, et al. (2001). "Intracervical fibrin sealants: a potential treatment for early preterm premature rupture of the membranes." Am J Obstet Gynecol **184**(3): 368-373.

Sener, T., S. Ozalp, et al. (1997). "Maternal blood clot patch therapy: a model for postamniocentesis amniorrhea." Am J Obstet Gynecol **177**(6): 1535-1536.

Sideris, I. G. and K. H. Nicolaides (1990). "Amniotic fluid pressure during pregnancy." Fetal Diagn Ther **5**(2): 104-108.

Simhan, H. N. and T. P. Canavan (2005). "Preterm premature rupture of membranes: diagnosis, evaluation and management strategies." BJOG **112 Suppl 1**: 32-37.

Skinner, S. J., G. A. Campos, et al. (1981). "Collagen content of human amniotic membranes: effect of gestation length and premature rupture." Obstet Gynecol **57**(4): 487-489.

Spitz, B., C. Vossen, et al. (1999). "Rupture of membranes before 26 weeks of gestation: outcome of 148 consecutive cases." J Perinat Med **27**(6): 451-457.

Strauss, J. F., 3rd (2012). "Extracellular Matrix Dynamics and Fetal Membrane Rupture." Reprod Sci.

Strohl, A., D. Kumar, et al. (2010). "Decreased adherence and spontaneous separation of fetal membrane layers--amnion and choriodecidua--a possible part of the normal weakening process." Placenta **31**(1): 18-24.

Teodoro, W. R., D. Andreucci, et al. (1990). "Short communication: placental collagen and premature rupture of fetal membranes." Placenta **11**(6): 549-551.

Toppozada, M. K., N. A. Sallam, et al. (1970). "Role of repeated stretching in the mechanism of timely rupture of the membranes." Am J Obstet Gynecol **108**(2): 243-249.

Vadillo-Ortega, F., G. Gonzalez-Avila, et al. (1995). "92-kd type IV collagenase (matrix metalloproteinase-9) activity in human amniochorion increases with labor." Am J Pathol **146**(1): 148-156.

Vadillo-Ortega, F., G. Gonzalez-Avila, et al. (1990). "Collagen metabolism in premature rupture of amniotic membranes." Obstet Gynecol **75**(1): 84-88.

Vadillo-Ortega, F., A. Hernandez, et al. (1996). "Increased matrix metalloproteinase activity and reduced tissue inhibitor of metalloproteinases-1 levels in amniotic fluids from pregnancies complicated by premature rupture of membranes." Am J Obstet Gynecol **174**(4): 1371-1376.

Vergani, P., L. Patane, et al. (2000). "Risk factors for neonatal intraventricular haemorrhage in spontaneous prematurity at 32 weeks gestation or less." Placenta **21**(4): 402-407.

Vettraino, I. M., J. Roby, et al. (1996). "Collagenase-I, stromelysin-I, and matrilysin are expressed within the placenta during multiple stages of human pregnancy." Placenta **17**(8): 557-563.

Ville, Y., K. Hecher, et al. (1998). "Endoscopic laser coagulation in the management of severe twin-to-twin transfusion syndrome." Br J Obstet Gynaecol **105**(4): 446-453.

Vintzileos, A. M., W. A. Campbell, et al. (1985). "Degree of oligohydramnios and pregnancy outcome in patients with premature rupture of the membranes." Obstet Gynecol **66**(2): 162-167.

Waite, J. H. (1999). "Reverse engineering of bioadhesion in marine mussels." Ann N Y Acad Sci **875**: 301-309.

Wang, H., S. Parry, et al. (2004). "Functionally significant SNP MMP8 promoter haplotypes and preterm premature rupture of membranes (PPROM)." Hum Mol Genet **13**(21): 2659-2669.

Winn, H. N., M. Chen, et al. (2000). "Neonatal pulmonary hypoplasia and perinatal mortality in patients with midtrimester rupture of amniotic membranes--a critical analysis." Am J Obstet Gynecol **182**(6): 1638-1644.

Wittenberg, G. F. (2011). "Elastic properties and yield stress of fetal membranes." Conf Proc IEEE Eng Med Biol Soc **2011**: 2123-2126.

Woessner, J. F., Jr. (1991). "Matrix metalloproteinases and their inhibitors in connective tissue remodeling." FASEB J **5**(8): 2145-2154.

Yamada, K., T. Chen, et al. (2000). "Chitosan based water-resistant adhesive. Analogy to mussel glue." Biomacromolecules **1**(2): 252-258.

Yeh, L. K., W. L. Chen, et al. (2005). "Soluble lumican glycoprotein purified from human amniotic membrane promotes corneal epithelial wound healing." Invest Ophthalmol Vis Sci **46**(2): 479-486.

Young, B. K., A. P. Mackenzie, et al. (2004). "Endoscopic closure of fetal membrane defects: comparing iatrogenic versus spontaneous rupture cases." J Matern Fetal Neonatal Med **16**(4): 235-240.

Young, B. K., A. S. Roman, et al. (2004). "The closure of iatrogenic membrane defects after amniocentesis and endoscopic intrauterine procedures." Fetal Diagn Ther **19**(3): 296-300.

Zisch, A. H. and R. Zimmermann (2008). "Bioengineering of foetal membrane repair." Swiss Med Wkly **138**(41-42): 596-601.

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8/2007 – 3/2008	Master Thesis at the Department of Cardiovascular Research, Inselspital, Berne, Group of Prof. Dr. med. H. Tevaearai "Custom Design of Microenvironment for Cardiac Tissue Engineering"
3/2006 – 7/2007	MSc in Biomedical Engineering with Specialization in Musculoskeletal System, Medical Faculty, University of Berne
8/2003 – 3/2004	Diploma Thesis at the Department of Immunology, The Scripps Research Institute, La Jolla, CA, USA, Group of Prof. M.D. B. Beutler "Positional Cloning of ADD, a Neurobehavioral Mutation in Mice", Main Focus on Genetics.
10/2000 – 3/2004	Academic Education at the Department of Biotechnology, University of Applied Sciences, Wädenswil, Switzerland

Professional Education & Work Experience

3/2006 – 8/2008	Part Time as Scientific Research Associate, Histology, Department of Anatomy, University of Berne, Switzerland, Group of Prof. Dr. P. Gehr
5/2004 – 6/2005	Biotechnologist at Berna Biotech Ltd., Assistant in a Project of Cancer Cell Vaccination used for Clinical Trials in GMP-Manufacturing, Responsible for the Downstream Processing
12/2000 – 12/2002	Part-time Employment as Night Manager in the Hotel Comfort Inn Royal, Zürich, Switzerland
8/1999 – 9/2000	Lab Technicians at the Department of Cell Biology, Swiss Federal Institute of Technology, Zürich, Switzerland, Group of Prof. Dr. U. Suter
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8/1996 – 7/1999	Swiss Technical Maturation
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Academic Awards

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Scientific Publications

September 2008	Du X, Schwander M, Moresco EM, Viviani P, Haller C, Hildebrand MS, Pak K, Tarantino L, Roberts A, Richardson H, Koob G, Najmabadi H, Ryan AF, Smith RJ, Müller U, Beutler B. (2008) "A catechol-O-methyltransferase that is essential for auditory function in mice and humans." Proc Natl Acad Sci, 105(38).
January 2010	Bilic G., Brubaker C., Messersmith P.B., Mallik A.S., Quinn T.M., Haller C., Done E., Gucciardo L., Zeisberger S.M., Zimmermann R., Deprest J., Zisch A.H. (2010) "Injectible candidate sealants for fetal membrane repair: bonding and toxicity in vitro", AJOG, 202(1).
January 2011	Haller C., Buerzle W., Brubaker C.E., Messersmith P.B., Mazza E., Ochsenbein-Koelble N., Zimmermann R., Ehrbar M. (2011) "Mussel-mimetic tissue adhesive for fetal membrane repair: a standardized ex vivo evaluation using elastomeric membranes", Prenat Diagn, 31(7).
Submitted	Haller C., Buerzle W., Mallik A.S., Mazza E., Ochsenbein-Koelble N., Zimmermann R., Ehrbar M. "Biomechanical characterizations of the fetal membranes rupture strength and its relation to microstructural components", AJOG.
Submitted	Haller C., Buerzle W., Brubaker C., Messersmith P.B., Mazza E., Ochsenbein-Koelble N., Zimmermann R., Ehrbar M. "Ex vivo repair of fetal membrane defects by the mussel-mimetic tissue adhesive", AJOG.

Oral Presentations

Mai 2009	Annual conference of perinatal medicine, Berlin, Germany
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September 2009	Annual conference of the European Society for Biomaterials (ESB), Lausanne, Switzerland

December 2009	Jahresschlussfortbildung, Frauenklinik, University Hospital Zürich, Switzerland
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